JOURNAL OF

Insect Physiology

VOLUME 3 1959

OL.
3
959

EDITORS:

V. G. DETHIER PHILADELPHIA **H. E. HINTON** BRISTOL **M. LÜSCHER** BERN

Editorial Advisory Board

Australia
D. F. WATERHOUSE

France
R. CHAUVIN P. JOLY

Germany
H. AUTRUM K. VON FRISCH B. HASSENSTEIN A. KÜHN

Holland
J. DE WILDE

Italy
C. JUCCI L. PARDI

Japan
S. FUKUDA

Switzerland
E. HADORN

United Kingdom
W. H. THORPE V. B. WIGGLESWORTH

U.S.A.
L. E. CHADWICK A. G. RICHARDS K. D. ROEDER C. M. WILLIAMS

U.S.S.R.
A. S. DANILYEVSKY

PERGAMON PRESS · *NEW YORK LONDON PARIS LOS ANGELES*

Notes for Contributors

I. GENERAL

1. Papers offered for publication should be original contributions in the field of insect physiology or be concerned with similar physiological problems in other groups of arthropods. Anatomical papers and papers on new techniques and methods may be accepted if they contribute to the solution of physiological problems.

2. Papers should be submitted to the appropriate editor:
PROF. V. G. DETHIER *Zoological Laboratory, University of Pennsylvania, Philadelphia* 4, *Pa., U.S.A.*
DR. H. E. HINTON *Department of Zoology, University of Bristol, England*
PROF. M. LÜSCHER *Zoologisches Institut der Universität Bern, Sahlistrasse* 8, *Bern, Switzerland*

3. Fifty free reprints of each paper are supplied. Additional copies may be purchased if they are ordered when the first proofs are returned. A reprint order form will be supplied with the galley proofs.

II. SCRIPT REQUIREMENTS

1. Papers must be typewritten (double spacing) on one side of the paper only. Tables and legends should be on separate sheets, and the place where they are to be inserted in the text should be indicated on the galley proofs. All papers must be provided with a brief abstract. Papers in French or German should be submitted with English abstracts. It will be appreciated if authors clearly indicate any Greek characters. To conserve space, authors are requested to mark less important portions of the papers, such as descriptions of methods, records of experimental results, etc. for printing in smaller type. The technical description of methods should be given in detail only when such methods are new. Authors will receive proofs for correction when their contributions are first set; page proofs will be sent only when the amount of alteration makes it advisable.

2. Illustrations should accompany the script separately, and legends should be kept to a minimum. Line-drawings or good photo-prints may be provided. Line drawings should include all relevant details: if they are already well drawn it may be possible to reproduce them direct from the originals. It is not possible to reproduce from prints with weak lines. Illustrations for reproduction should normally be about twice the final size required. Half-tones should be included only where they are essential. Any lettering on diagrams—other than Greek letters, symbols, etc.—should be stencilled and should be sufficiently large and bold to permit reproduction when the diagram has been reduced to a size suitable for inclusion in the journal.

3. Tables and figures should be constructed so as to be intelligible without reference to the text. Every table should be provided with an explanatory caption and each column should carry an appropriate heading. Units of measure should always be clearly indicated. The same data should not be published in tables and figures. The following standard characters may be used:

▲ △ ⊡ □ ● ○ △ ◌ ⊕ ⊖ ◔ ⊗.

4. References should be quoted in the text as follows: EYSENCK (1947). References should be listed at the end of the paper and not given as footnotes. They should be arranged in alphabetical order as follows:

EYSENCK H. J. (1947) *Dimensions of Personality*. Routledge & Kegan Paul, London.
FRENCH J. D., VERZEANO M. and MAGOUN H. W. (1953) A neural basis of the anaesthetic state. *Arch. Neurol. Psychiat.* **69**, 591–600.
WIGGLESWORTH V. B. (1952) The thoracic gland in *Rhodnius prolixus* (Hemiptera) and its role in moulting. *J. exp. Biol.* **29**, 561–570.

Titles of periodicals cited are to be abbreviated as in the *World List of Scientific Periodicals, 3rd edition*, 1952.

Publishing Offices: 122 *East 55th Street, New York* 22, *N.Y.* (*Plaza* 3–9651)

Issued in parts. Subscription (*A*) *per volume* $20 (£7) *including postage;* (*B*) *For subscribers who notify the Publisher direct certifying that the journal is for their private use* $15 (£5 5*s.*) *per annum including postage.*

Two volumes per annum

Back numbers $24 (£8) *per volume*

Subscriptions should be sent to:
Pergamon Press, Inc., 122 *East 55th Street, New York* 22, *N.Y.*

Copyright © 1959 *Pergamon Press Ltd.*

PERGAMON PRESS, INC.

122 EAST 55TH STREET, NEW YORK 22, N.Y. 4 & 5 FITZROY SQUARE, LONDON W.1

Contents of Volume 3

Vol. 3, No. 1 PAGE

LAMB, K. P.: Composition of the honeydew of the aphid *Brevicoryne brassicae* (L.) feeding on swedes (*Brassica napobrassica* DC.) 1

DAVID, W. A. L.: The systemic insecticidal action of paraoxon on the eggs of *Pieris brassicae* (L.) 14

MOROHOSHI, SEIJIRO: Hormonal studies on the diapause and non-diapause eggs of the silkworm, *Bombyx mori* L. 28

NAKA, K. and KUWABARA, M.: Electrical response from the compound eye of *Lucilia* 41

CRONE-GLOOR, URSULA VON DER: Quantitative untersuchung der Freien Aminosäuren und Polypeptide während der Embryonalentwicklung von *Drosophila melanogaster* 50

AUCLAIR, JACQUES L.: Amino acid oxidase activity in the fat body and Malpighian tubes of some insects 57

HUDSON, BRUCE W., BARTEL, ALLEN H., and CRAIG, RODERICK: Pteridines in the milkweed bug, *Oncopeltus fasciatus* (Dallas)—II. Quantitative determination of pteridine content of tissues during growth 63

NOTICE 74

Vol. 3, No. 2

WILDE, J. DE, DUINTJER, C. S., and MOOK, L.: Physiology of diapause in the adult Colorado beetle (*Leptinotarsa decemlineata* Say)—I. The photoperiod as a controlling factor 75

LASKER, REUBEN: Utilization and excretion of organic nitrogen by the silverfish, *Ctenolepisma lineata* 86

LEES, A. D.: The role of photoperiod and temperature in the determination of parthenogenetic and sexual forms in the aphid *Megoura viciae* Buckton—I. The influence of these factors on apterous virginoparae and their progeny 92

FRIEDMAN, STANLEY: Sustained flight in *Phormia* (by a new method) and its effect on blood pH 118

GRAF, G. E., HADORN, E., and URSPRUNG, H.: Experiments on the *iso*xanthopterin metabolism in *Drosophila melanogaster* 120

BROWN, R. H. J.: A simple activity recorder 125

AUCLAIR, JACQUES L.: The influence of dietary amino acids on the blood amino acids of the German cockroach, *Blattella germanica* (L.) 127

OHNISHI, EIJI: Pigment composition in the pupal cuticles of two colour types of the swallowtails, *Papilo xuthus* L. and *P. protenor demetrius* Cramer 132

YAMASAKI, TERUO and NARAHASHI, TOSHIO: The effects of potassium and sodium ions on the resting and action potentials of the cockroach giant axon 146

BÜCKMANN, DETLEF: Die Auslösung der Umfärbung durch das Häutungshormon bei *Cerura vinula* L. (Lepidoptera, Notodontidae) 159

PAPERS TO BE PUBLISHED IN FUTURE ISSUES 190

Vol. 3, No. 3

HOSOI, TERUHIKO: Identification of blood components which induce gorging of the mosquito 191

OHNISHI, EIJI: Studies on the mechanism of tyrosinase activation in the housefly, *Musca vicina* Macq. 219

YAMASAKI, TERUO and NARAHASHI, TOSHIO: Electrical properties of the cockroach giant axon 230

VOL. 3 1959

PAGE

MILES, P. W.: The salivary secretions of a plant-sucking bug, *Oncopeltus fasciatus* (Dall.) (*Heteroptera*: *Lygaeidae*)—I. The types of secretion and their roles during feeding 243
PATTON, R. L., GARDNER, J., and ANDERSON, ANN D.: The excretory efficiency of the American cockroach, *Periplaneta americana* L. 256
TREAT, ASHER E. and ROEDER, KENNETH D.: A nervous element of unknown function in the tympanic organs of moths 262
MARUYAMA, K.: Insect actomyosin. A flow birefringence study 271
LEVINSON, ZWI H. and BERGMANN, ERNST D.: Vitamin deficiencies in the housefly produced by antivitamins 293
ASPEREN, K. VAN: Distribution and substrate specificity of esterases in the housefly, *Musca domestica* L. 306
PAPERS TO BE PUBLISHED IN FUTURE ISSUES 323

Vol. 3, No. 4

LAMBREMONT, E. N.: A study of acid phosphatase of the mosquito *Aedes aegypti* L. 325
CHEN, P. S.: Studies on the protein metabolism of *Culex pipiens* L.—III. A comparative analysis of the protein contents in the larval haemolymph of autogenous and anautogenous forms 335
BALDWIN, W. F. and SALTHOUSE, T. N.: Dermal glands and mucin in the moulting cycle of *Rhodnius prolixus* Stål. 345
BHEEMESWAR, B. and FAULKNER, P.: Metabolism of oximes in the silkworm *Bombyx mori* L.—I. Inhibition of transaminases 349
MORIARTY, F.: The 24-hr rhythm of emergence of *Ephestia kühniella* Zell. from the pupa 357
JOHNSON, B.: Studies on the degeneration of the flight muscles of alate aphids—II. Histology and control of muscle breakdown 367
HOYLE, G.: The neuromuscular mechanism of an insect spiracular muscle 378
HESLOP, J. P. and RAY, J. W.: The reaction of the cockroach *Periplaneta americana* L. to bodily stress and DDT 395
CARRINGTON, C. B. and TENNEY, S. M.: Chemical constituents of haemolymph and tissue in *Telea polyphemus* Cram. with particular reference to the question of ion binding 402
PAPERS TO BE PUBLISHED IN FUTURE ISSUES 414

Author Index

ANDERSON A. D. p. 256
ASPEREN K. VAN p. 306
AUCLAIR J. L. pp. 57, 127
BALDWIN W. F. p. 345
BARTEL A. H. p. 63
BERGMANN E. D. p. 293
BHEEMESWAR B. p. 349
BROWN R. H. J. p. 125
BÜCKMANN D. p. 159
CARRINGTON C. B. p. 402
CHEN P. S. p. 335
CRAIG R. p. 63
CRONE-GLOOR U. VON DER p. 50
DAVID W. A. L. p. 14
DUINTJER C. S. p. 75
FAULKNER P. p. 349
FRIEDMAN S. p. 118
GARDNER J. p. 256
GRAF G. E. p. 120
HADORN E. p. 120
HESLOP J. P. p. 395
HOSOI T. p. 191
HOYLE G. p. 378
HUDSON B. W., p. 63
JOHNSON B. p. 367
KUWABARA M. p. 41
LAMB K. P. p. 1
LAMBREMONT E. N. p. 325
LASKER R. p. 86
LEES A. D. p. 92
LEVINSON Z. H. p. 293
MARUYAMA K. p. 271
MILES P. W. p. 243
MOOK L. p. 75
MORIARTY F. p. 357
MOROHOSHI S. p. 28
NAKA K. p. 41
NARAHASHI T. pp. 146, 230
OHNISHI E. pp. 132, 219
PATTON R. L. p. 256
RAY J. W. p. 395
ROEDER K. D. p. 262
SALTHOUSE T. N. p. 345
TENNEY S. M. p. 402
TREAT A. E. p. 262
URSPRUNG H. p. 120
WILDE J. DE p. 75
YAMASAKI T. pp. 146, 230

VOL.
3
1959

VO
3
19

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 1 *to* 13. *Pergamon Press Ltd., London. Printed in Great Britain*

COMPOSITION OF THE HONEYDEW OF THE APHID *BREVICORYNE BRASSICAE* (L.) FEEDING ON SWEDES (*BRASSICA NAPOBRASSICA* DC.)

K. P. LAMB

Plant Diseases Division, Department of Scientific and Industrial Research, Auckland, New Zealand

(*Received* 25 *April* 1958)

VOL. 3 1959

Abstract—Honeydew of *Brevicoryne brassicae* feeding on swedes was a faintly acid solution (*p*H 5·1–7·4) containing approximately 11 per cent dry matter, mostly carbohydrate in nature (88 per cent carbohydrate, 7 per cent nitrogenous compounds). Reducing sugars (fructose and glucose) comprised 27 per cent of the total carbohydrate. Acid hydrolysis of the non-reducing sugars yielded mainly glucose and a little fructose. Free amino acids and amides (in more or less equal amounts) accounted for 72–90 per cent of the total-N. Glutamine was the major constituent while glutamic and aspartic acids and two unknown ninhydrin-reacting compounds "under glutamine" also occurred in moderately large amounts. Smaller quantities of seventeen other amino acids were also present. All the amino acids and amides found in the honeydew also occurred in soluble form in the swede leaf in similar proportions. Nitrates accounted for 5 per cent of the total-N and ammonia appeared in trace amounts. All but 5–8 per cent of the total-N could be accounted for. Uric acid, urea, allantoin, allantoic acid, creatine, or creatinine could not be detected. Limits of detection : uric acid 1 per cent, urea 2½ per cent of total-N. Citric acid was the major organic acid but malic, succinic, and fumaric acids occurred in smaller amounts. Chlorides and phosphates were present, 2·8 per cent of the dry matter being phosphorus (expressed as P_2O_5). The N/P ratio of the honeydew (0·71) was considerably lower than that of the soluble fraction of the swede leaf (3·4) or of whole alate *B. brassicae* (8·2). The only classical form of "excretory nitrogen" found was ammonia which comprised less than 0·5 per cent of the total nitrogen. It is hypothesized that ammonia may be detoxified in the mycetome by symbiotic organisms which utilize it preferentially as a nitrogenous substrate.

INTRODUCTION

MANY plant-feeding Homoptera (especially aphids and scale insects) produce copious quantities of clear, fluid excreta. This sweet, sticky honeydew was once believed to be exuded from leaves or from aphid cornicles but it is now known to be released in droplets from the anus of the insect concerned.

Honeydew contains much carbohydrate and appreciable quantities of amino and other organic compounds. It plays an important biological role as food for many insects, especially Hymenoptera. Zoebelein (1956) recorded 246 species of honeydew-feeding insects belonging to 49 families. Some are obligate honeydew feeders (certain ants), while others are facultative in that respect. The latter group includes many adult insect parasites (Ichneumonids, Tachinids) and

predators (Coccinellids, Anthribids, and Syrphids). Bees and wasps sometimes feed on honeydew, and honeydew honey is sometimes of considerable economic importance as, for example, in parts of Turkey (ERMIN, 1950). However, honey containing large amounts of honeydew sometimes has undesirable physical properties, and cases are known of the production of poisonous honey, e.g. through the incorporation of honeydew of *Scolypopa australis* Walk. which was feeding on the poisonous plant *Coriaria arborea* (PALMER-JONES *et al.*, 1947). Certain honeydews provide the substrate for growth of sooty mould fungi on plants. Although these fungi are not normally regarded as pathogenic they are unsightly and may spread over leaves to such an extent as to interfere with photosynthesis of the host.

So long as an aphid is feeding, it constantly excretes droplets of honeydew. There has been much speculation as to the reason for the production of honeydew in such large quantities. Early workers believed that aphid food contained little nitrogenous material and that consequently large volumes of food had to be ingested to obtain sufficient amino-nitrogen. More recent work has shown that substantial quantities of a broad spectrum of amino acids and amides are excreted unchanged by aphids so that the earlier view is now untenable. This subject was recently reviewed by WATERHOUSE (1957) who concluded that "honeydew must, therefore, be regarded as a mixture of excess carbohydrates, amino acids, water, and other sap constituents to which the insect contributes various additional metabolic and excretory products."

Little is known of the nature of these metabolic and excretory products. In fact, little is known of the nutritional requirements of phytophagous insects in general (HOUSE, 1956). Studies of aphid nutrition have been hindered in the past through inability to feed aphids artificially. KENNEDY and MITTLER (1953) succeeded in sampling aphid food (phloem sap) by way of stylets cut *in situ*. This technique works quite well with large aphids on woody plants but with smaller aphids on herbaceous plants it has not proved possible to collect sufficient quantities of phloem sap for comprehensive quantitative analyses. LINDEMANN (1948) sampled phloem sap from cut twigs of currants and followed seasonal variations in total nitrogen content. Marked changes were found corresponding to phases of plant growth, the concentration being highest in spring when growth was most active. Concurrent analyses of honeydew of *Cryptomyzus ribis* showed the same trend. MITTLER (1953) confirmed this with *Tuberolachnus salignus* on willows and showed that the greatest variety and abundance of amino acids in sap and honeydew occurred during vigorous growth and senescence of foliage. Aphid food (phloem sap) and excreta therefore vary in composition according to the state of growth of the host plant.

In view of the nutritional importance of nitrogenous compounds (both for aphids and honeydew feeders), and as part of a programme for the investigation of the nature of aphid resistance, the honeydew of the cabbage aphid (*Brevicoryne brassicae* (L.)) feeding on swede plants (*Brassica napobrassica* DC.) has been examined with special reference to these compounds.

MATERIAL AND METHODS

Fresh honeydew was collected from aphid colonies on swede leaves (*Brassica napobrassica* DC.) with the aid of a capillary pipette. Daily collections were weighed, frozen, and stored at −10°C until required. In some cases honeydew was collected on glass plates beneath aphid colonies, rapidly dried *in vacuo*, weighed, and stored at −10°C.

Paper chromatography of amino acids was by the method of DENT (1947). Phenol : water and *n*-butanol : acetic acid : water (4 : 1 : 5) were the main solvents used. Amino acids were detected with 1 per cent ninhydrin in 95 per cent ethanol and/or 0·2 per cent isatin in acetone.

Organic acids were detected with 0·1 per cent brom-cresol-green in ethanol on ascending chromatograms. As well as the above solvents, *n*-butanol : formic acid : water (4 : 1 : 5) and ether : acetic acid : water (13 : 3 : 1) were used (LUGG and OVERELL, 1947; DENISON and PHARES, 1952). Sugars were chromatographed in *n*-butanol : acetic acid : water (4 : 1 : 5) (PARTRIDGE and WESTALL, 1948) or *n*-butanol : ethanol : acetone : water (5 : 4 : 3 : 2) (GRAY and FRAENKEL, 1954). The identification of sugars was checked by paper electrophoresis with borate buffer *p*H 9·0, using the apparatus of MARKHAM and SMITH (1952). For detection of sugars the benzidine-trichloracetate reagent of BACON and EDELMAN (1951) was used. Periodate oxidation followed by the ammoniacal silver nitrate procedure of TREVELYAN *et al.* (1950) was used for detection of trehalose (EVANS and DETHIER, 1957). Ketohexoses were detected with naphthoresorcinol-HCl (FORSYTH, 1948). Anilin-phthalate was used for aldohexoses (PARTRIDGE, 1949). Eluted spots from chromatograms were acid hydrolysed in 1·5N sulphuric acid (PIRT and WHELAN, 1951) and re-chromatographed. Standards of authentic organic acids or sugars were run on all chromatograms or in paper electrophoresis.

Because the high sugar content of honeydew often interfered with the chromatographic separation of other constituents, samples were first fractionated on columns of ion exchange resins. Two 5×350 mm columns were used in series. The first column, containing the cation exchange resin Zeokarb 215-H, extracted amino acids, amides and bases from dilute honeydew solution. The second column, of anion exchange resin (IRA 400-OH), removed organic acids from solution, leaving the sugars in the final effluent. Amino acids were eluted with 5N- and N-ammonium hydroxide while organic acids were eluted from the second column with N-ammonium carbonate. Ammonia was boiled off the eluates which were concentrated to a convenient volume on a water bath. Part of the amino acid fraction was hydrolysed with N- and 6N-HCl prior to chromatography. Comparison of honeydew samples before and after ion exchange treatment showed that there was some loss of arginine on the cation exchange column but otherwise there was no apparent change in composition.

Total nitrogen was determined by the micro-Kjeldahl procedure of MARKHAM (1942). Ammonia was isolated by microdiffusion (CONWAY, 1950) and estimated by the phenol-hypochlorite method of RUSSELL (1944). Glutamine and asparagine were determined by the method of PUCHER *et al.* (1935). Total amide, nitrate, and

VOL. 3 1959

nitrite nitrogen were determined by the method of VARNER *et al.* (1953). The ninhydrin method of COCKING and YEMM (1954) was used for α-amino nitrogen estimation before and after acid hydrolysis (LAMB, 1957).

Total carbohydrate was estimated by the anthrone method of YEMM and WILLIS (1954). Reducing sugars were determined by the method of SOMOGYI (1945), using the arsenomolybdate reagent of NELSON (1944) for colour development.

Phosphate was detected on chromatograms by the HANES-ISHERWOOD (1949) reagent and was determined quantitatively in honeydew by the method of ALLEN (1940) following sulphuric acid digestion (JONES *et al.*, 1951).

RESULTS

Honeydew is excreted from the anus of the cabbage aphid as droplets of clear, colourless, viscous fluid, coated externally with white, powdery wax similar to that coating the body. The water-repellent nature of the waxy coating results in droplets remaining discrete and relatively uncontaminated about the aphid colony. By using a sufficiently fine capillary to collect the honeydew, the waxy powder was filtered off during collection.

Fresh honeydew contained 5·9–17·7 per cent dry matter (mean 11·4 per cent six collections). The *p*H varied from 5·1–7·4 and conductivity measurements on two samples indicated an electrolyte content equivalent to 1–6 per cent KCl (based on dry matter). Most of the dry matter was composed of carbohydrates (about 90 per cent by weight). Nitrogenous compounds comprised about 3–9 per cent of the dry matter. The major constituents of *B. brassicae* honeydew are considered below.

(I) *Nitrogenous fraction*

The results of quantitative analysis of three separate honeydew collections are summarized in Table 1. Total-N of the honeydew varied from 0·16 to 0·32 per cent of the fresh weight. In four dried samples it comprised 0·44–1·8 per cent of the dry weight. Amino- and amide-N accounted for 72–90 per cent of the total-N. In the first and third sets of analyses all but 5–8 per cent of the nitrogen could be accounted for though there was discrepancy of 28 per cent in the second sample.

The principal nitrogenous constituent of honeydew was glutamine, which accounted for approximately two-thirds of the total-N. Other important amino constituents were glutamic acid, aspartic acid, and two or three unknown ninhydrin-reacting compounds running under glutamine in phenol and butanol-acetic solvents. One of these compounds gave a brownish-pink reaction with ninhydrin and all were unstable to acid hydrolysis (N or 6N-HCl) or to peroxide oxidation. An increase in glycine and alanine was also noted on acid hydrolysis or peroxide oxidation.

Chromatographic estimation of the major amino acid constituents of two samples of fresh honeydew by the ultramicro method of AUCLAIR and DUBREUIL

(1952) confirmed that glutamine was by far the most abundant amino-N constituent (see Table 2). The amino acid content of the first sample (collected in summer) was approximately five times that of the second sample (collected in winter).

TABLE 1—NITROGENOUS CONSTITUENTS OF HONEYDEW (3 COLLECTIONS)

	Collection 1 (11 August 1952)			Collection 2 (11 August 1953)			Collection 3 (11 July 1956)		
	% w/w fresh h.d.	% w/w dry h.d.	as % total-N	% w/w fresh h.d.	% w/w dry h.d.	as % total-N	% w/w fresh h.d.	% w/w dry h.d.	as % total-N
Total-N	0·326	—	100	0·159	1·45	100	—	0·44	100
Ammonia-N	0·035	—	11*	0	0	0	—	0·001	0·2
Glutamine amide-N	0·065	—	20*	0·053	0·48	33	—	0·22	49
Asparagine amide-N	0·053	—	16	0·001	0·01	8	—	0·22	49
α-amino-N	0·148	—	45	0·050	0·46	31	—	0·18	41
Nitrite + nitrate-N	—	—	—	—	—	—	—	0·02	5
Undetermined N	0·025	—	8	0·055	0·51	28	—	0·02	5
Total amino acids and amides (N × 7·2)†	2·35			1·14	10·4			3·17	

* Some decomposition of glutamine in this sample resulted in a lowered glutamine figure and an elevated value for ammonia.

† Conversion factor calculated from approximate amino acid composition.

Amino acids occurring in moderate amounts included serine, glycine, threonine, alanine, valine, methionine, leucines, arginine, lysine, and cysteic acid. Sometimes, in trace amounts, tyrosine, γ-aminobutyric acid, phenylalanine,

TABLE 2—QUANTITATIVE AMINO ACID DETERMINATIONS ON TWO FRESH HONEYDEW SAMPLES

	Collection of 6 February 1953		Collection of 23 July 1953
	mg/g fresh h.d.	mg/g dry h.d.	mg/g fresh h.d.
Glutamine	>5·0	>43·7	2·19
Glutamic acid	2·0	17·5	0·42
Aspartic acid	4·0	35·0	0·74
Valine/Methionine	—	—	0·36
Leucine/*Iso*-leucine	0·3	2·5	—

dihydroxyphenylalanine, histidine, cystine, and β-alanine were also present. With the exception of dihydroxyphenylalanine, all the above compounds were found in alcoholic extracts of swede leaves.

There was no indication of the presence of protein or substantial amounts of peptide in honeydew—unless the "under glutamine" spots were peptides—since acid hydrolysis did not result in a substantial increase in amount of any amino acids (apart from glycine and alanine referred to above, and the conversion of glutamine and asparagine to glutamic acid and aspartic acid respectively). No turbidity resulted on the addition of ethanol or trichloracetic acid to honeydew.

Qualitative tests did not reveal the presence of the following nitrogen compounds. (Limits of detection as percentage total nitrogen are given in each case.) Urea (2½ per cent; Ehrlich's reagent, urease); allantoin or allantoic acid (5 per cent; Ehrlich's reagent); creatine or creatinine (2 per cent; alkaline picric acid, ferricyanide-nitroprusside reagent of BERRY *et al.*, 1951); or uric acid (1 per cent; ammoniacal silver nitrate, ultraviolet absorption spectrum, chromatography by the method of MARKHAM and SMITH, 1949, selective precipitation by the method of BERGMANN and DIKSTEIN, 1954).

(II) *Organic acids*

Citric acid was the major organic acid present in honeydew. Smaller amounts of malic, succinic, and fumaric acids were also present. Traces of pyrrolidone carboxylic acid (a breakdown product of glutamic acid), *iso*citric acid, and several unidentified acids were also found in some samples. The presence of phosphate, nitrate, chloride, and sulphur was confirmed by spot tests (FEIGL, 1947).

In swede leaf extracts malic acid was present in larger amounts than citric acid. This could possibly be accounted for by diurnal variation since swede leaf samples were taken only in the daytime while honeydew included 24 hour samples.

(III) *Carbohydrates*

On chromatograms, much glucose and some fructose were found consistently in honeydew samples. Traces of sucrose were found in two samples. Some

TABLE 3—QUANTITATIVE ANALYSIS OF CARBOHYDRATE, HONEYDEW COLLECTION 3 (OF TABLE 1); VALUES EXPRESSED AS GLUCOSE PER CENT DRY MATTER

	Per cent dry matter	Per cent total carbohydrate
Total carbohydrate (anthrone)	88 ± 0·65	—
Reducing sugars (Somogyi-Nelson)	24 ± 0·65	27
Reducing sugars (after acid hydrolysis)	82 ± 2·68	—
Non-reducing carbohydrate (by difference)	64	73

oligosaccharides or higher molecular weight compounds streaked from the origin when large samples were taken and these compounds reacted only weakly with the usual spraying reagents.

Quantitative analysis of honeydew (Table 3) indicated the presence of substantial quantities of non-reducing carbohydrate (64 per cent of the dry matter) not revealed on earlier chromatograms.

Swede leaf extracts contained much fructose, glucose, and sucrose and little raffinose. The substantial decrease in sucrose in honeydew indicates invertase activity in the aphid gut.

(IV) *Other constituents*

Quantitative analysis of one bulk collection of honeydew (collection 3, Table 1) for phosphorus gave a value of 0·62 per cent (based on dry weight), i.e. 2·84 per cent as P_2O_5. The N/P ratio for this honeydew was 0·71. The N/P ratio of the alcohol soluble fraction of swede leaf tissue was found to be 3·4, whereas the N/P ratio of whole alate *B. brassicae* was found to be 8·2.

DISCUSSION

Two questions emerge from this work: (*a*) does honeydew largely comprise undigested food, and (*b*) in what form is nitrogen excreted after being subjected to the metabolic processes of the insect?

The qualitative similarity of the amino acid and amide constituents of honeydew and the soluble fraction of swede leaves suggests that honeydew is at least partly composed of undigested sap as WATERHOUSE (1957) has indicated. MITTLER (1953) found no evidence for selective absorption of amino acids in the gut of *Tuberolachnus salignus*. These findings are confirmed for the cabbage aphid but direct chromatographic comparison is not sufficiently sensitive to detect minor quantitative changes in individual amino acid constituents. Precise estimation of individual amino acids in both food and excreta will be required before this is firmly established.

Fluctuations in the amino acid content of the honeydew closely follow seasonal fluctuations of plant amino acids associated with plant growth. For example, *B. brassicae* honeydew collected in summer when plants were actively growing in the glasshouse contained five times the quantity of amino acids found in winter (Table 2) when plant growth was almost at a standstill. This variation is of the same order as the seasonal variation in currant phloem sap nitrogen (LINDEMANN, 1948).

The carbohydrate fraction of *B. brassicae* honeydew has not been analysed in detail. The reducing sugars (27 per cent of the total carbohydrate) were identified chromatographically and electrophoretically as fructose and glucose. Comparative values for reducing sugar content of a number of honeydews are given in Table 4. The value found for cabbage aphid honeydew is very close to the average value. It is probable that these sugars, at least, represent surplus undigested food.

The composition of the non-reducing sugars is more complex. Earlier workers recorded the presence of large quantities of dextrin (BOUSSINGAULT, 1872; ARNHART, 1927; GEINITZ, 1930; MICHEL 1942), or melezitose (MICHEL, 1942).

DUSPIVA (1953) reported the presence of complex mixtures of oligosaccharides in honeydew. He postulated that these were synthesized enzymatically in the aphid gut by transglycosidation. WHITE and MAHER (1953) showed that when invertase acted on sucrose *in vitro* various oligosaccharides were produced. In the same year, GRAY and FRAENKEL reported the presence of one of these, fructomaltose, in the honeydew of *Pseudococcus citri* and *Aphis spiraecola*. DUSPIVA (1954) stated that apart from the sugars glucose, fructose, and sucrose which were derived from the food, three oligosaccharide series occurred in honeydew as a

TABLE 4—REDUCING SUGAR CONTENT OF VARIOUS HONEYDEWS

	Expressed as per cent total sugar
Sinai manna (BOUSSINGAULT, 1872)	25
Honeydew on *Tilia* (BOUSSINGAULT, 1872)	26
Lachnus muravensis honeydew (ARNHART, 1927)	14
L. pichtae honeydew (GEINITZ, 1930)	48
L. pichtae honeydew (ERMIN, 1950)	31
L. roboris honeydew (MICHEL, 1942)	19
Pseudococcus brevipes honeydew (GRAY, 1952)	34
Myzus circumflexus honeydew (MALTAIS and AUCLAIR, 1952)	36
Brevicoryne brassicae honeydew (see Table 3)	27

result of transglycosidation in the gut. These were an "amylose series" (with 1, 4-linked hexose groups), a "dextran series" (with 1, 6-linked groups), and a third series derived from a terminal sucrose linked with one or more glucose units. He synthesized members of these oligosaccharide series *in vitro* using aphid gut and salivary gland preparations and various sugars as substrates. According to DUSPIVA these different groups of carbohydrates were represented in different amounts in the honeydew of different aphids. GRAY and FRAENKEL (1954) recorded the presence of glucose-l-phosphate and fructomaltose in the honeydew of *Pseudococcus citri*, *Pulvinaria vitis* and *Aphis spiraecola*. WOLF and EWART (1955) found oligosaccharides of DUSPIVA's third series in the honeydew of *Coccus hesperidum*, viz. glucosucrose (=fructomaltose), maltosucrose, maltotriosucrose, and ? malto-tetro-sucrose.

There seems little doubt that a substantial part of the carbohydrate present in honeydew is in the form of oligosaccharides synthesized in the gut from simple sugars ingested by the aphid.

Apart from traces of sucrose and raffinose (which probably represented undigested food) the non-reducing carbohydrates of *B. brassicae* were not identified. Melizotose was not found and attempts to isolate dextrin by acetone precipitation were not successful. Since sucrose was apparently digested by the aphid it is possible that oligosaccharides of DUSPIVA's third series comprised part of the non-reducing fraction.

In so far as extensive changes occur in the carbohydrate fraction during passage through the gut, the greater part of the honeydew sugars must be regarded as partially digested rather than undigested material.

When proteins or amino acids are digested by animals, ammonia is produced. This is usually converted into various nitrogenous compounds (uric acid, urea) before excretion, but is sometimes excreted unchanged, and this is correlated with ecological habitats. Thus terrestrial insects are mostly uricotelic since 80–90 per cent of the total nitrogen excreted is in the form of uric acid. This type of excretion is associated with a need for water conservation, especially during early development. Urea only occurs in small amounts in insect excreta. The excretion of ammonia, uncommon among terrestrial insects, is characteristic of aquatic organisms. This is because ammonia is a highly toxic compound and requires a plentiful supply of water for its efficient elimination.

The question now arises whether or not aphids conform to the normal uricotelic type of excretion most common in insects since the only classical form of excretory nitrogen found in honeydew of *B. brassicae* was ammonia, and this comprised less than 0·5 per cent of the total nitrogen in the excreta.

VOL. 3 1959

GRAY and FRAENKEL (1954) claimed that uric acid was present in the honeydew of the citrus mealybug since they obtained positive reactions with Benedict's, Schiff's and the murexide tests. Earlier, TRUSZKOWSKI and CHAJKINÓWNA (1935) were unable to detect uric acid, uricase, or xanthine oxidase in aphids. GRAY (1952) observed on his chromatograms of pineapple mealybug honeydew, the formation of two black spots with mercuric nitrate reagent for purines and pyrimidines. One of these spots coincided with cystine and the other was very near aspartic acid. Since these chromatograms were run in phenol and butanol-acetic acid-water it is unlikely that either spot was uric acid since this substance is usually found near the origin in those solvents. MALTAIS and AUCLAIR (1952) working with the honeydew of *Myzus circumflexus* were able to account for the whole of the nitrogen present as amides and amino acids. Good agreement was found between results obtained by quantitative paper chromatography, the Van Slyke gasometric method for amino nitrogen and the Kjeldahl method for total nitrogen.

The findings of GRAY and FRAENKEL conflict with those of MALTAIS and AUCLAIR and TRUSZKOWSKI and CHAJKINÓWNA. Further, the tests used by GRAY and FRAENKEL were not specific for uric acid alone. Benedict's arsenophosphotungstate reagent reacts with glucose (SNELL and SNELL, 1953), a common honeydew constituent. Schiff's test, based on the blackening of silver in the presence of uric acid under alkaline conditions, is unsuitable for use on raw biological extracts of this sort since it should not be applied in the presence of chlorides. In addition, a positive reaction can be obtained with a variety of reducing substances. The murexide test is given by pteridines (GATES, 1947) which are widely distributed in insects.

Careful examination of *B. brassicae* honeydew failed to reveal the presence of uric acid. Tests for urea, allantoin, allantoic acid, creatine, or creatinine were also negative. Extraction of whole aphids with boiling 80 per cent ethanol or

cold 0·1 N sodium hydroxide and chromatography of the extracts, failed to reveal the presence of uric acid. We may therefore assume that aphids are not uricotelic. This conclusion gains added weight from the fact that aphids lack Malpighian tubules (WEBER, 1929), which are primarily concerned in uric acid excretion in other insects (WIGGLESWORTH, 1931).

It is possible that other forms of excretory nitrogen were present in the 5–8 per cent of "undetermined-N" in the honeydew analyses (samples 1 and 3, Table 1) even though this discrepancy is probably within the experimental error of the analyses.

The low level of excretory nitrogen (ammonia) in the honeydew (assuming that the amide and amino-N represent undigested food rather than true excretory products) suggests either that aphid digestion is highly efficient or that excretory nitrogen is disposed of within the insect and stored in some undetermined form. On the other hand the relatively large volume of water passing through the gut may be sufficient to dilute and flush out ammonia and thus obviate its toxic effects; a situation parallel to that found in aquatic insects.

It might be expected that aphids would possess some internal arrangements for detoxicating part of the ammonia formed in digestion. The internal organ which comes to mind in this connexion is the mycetome which occupies a large part of the body cavity (especially in young, growing aphids).

The mycetomes are packed with symbiotic organisms which are present from the early stages of embryonic development. TÓTH (1940) drew attention to the mycetomes in aphids and suggested that they played a role in supplementing the protein intake by fixation of atmospheric nitrogen. TÓTH, WOLSKY, and BATYKA (1944) demonstrated the fixation of atmospheric nitrogen *in vitro* using a surviving system technique with 21 species of Rhynchota. This finding was not confirmed for the two species of aphids investigated by SMITH (1948).

FINK (1952) succeeded in growing the symbionts of *Pseudococcus citri* in culture and was able to demonstrate the fixation of atmospheric nitrogen and ammonia by the bacteria as well as the production of various amino acids, vitamins, and enzymes. He suggested that the insects were dependent upon the symbionts for the provision of important nutritional factors. The experimental evidence provided by this worker is more convincing than much of the earlier work in this field.

When nitrogen-fixing organisms are placed in a medium containing ammonia (or compounds that readily give rise to ammonia), fixation of atmospheric nitrogen immediately ceases (WILSON and BURRIS, 1947). Aphid haemolymph, which bathes the mycetomes, contains abundant organic nitrogen, and it would be surprising if fixation of atmospheric nitrogen could proceed under such conditions. Nitrogen-fixing organisms of the *Azotobacter* type are, however, capable of fixing ammonia in their substrate (GALE, 1951; FRY, 1955), and in fact, will fix ammonia preferentially in the presence of other nitrogenous compounds.

In view of the ammonotelic metabolism of aphids, the finding of FINK that the organisms of the mycetome can utilize ammonia assumes a special significance.

While the mycetomes may well provide essential vitamins and amino acids for the aphid, it seems not improbable that their major function is detoxification of part of the ammonia produced in digestion by utilizing it preferentially as a nitrogenous substrate for the enclosed symbionts.

REFERENCES

ALLEN R. J. L. (1940) The estimation of phosphorus. *Biochem. J.* **34**, 858–865.

ARNHART L. (1927) Österreichischer Lärchenhonigtau, Lärchenmanna und Lärchenhonig. *Z. angew. Ent.* **12**, 457–472.

AUCLAIR J. L. and DUBREUIL R. (1952) A simple ultramicromethod for the quantitative estimation of amino acids by paper partition chromatography. *Canad. J. Zool.* **30**, 109–113.

AUCLAIR J. L. and MALTAIS J. B. (1952) Occurrence of α-amino-butyric acid in extracts of *Pisum sativum* (L.) and in the honeydew of *Myzus circumflexus* (Buck.). *Nature, Lond.* **170**, 1114–1115.

BACON J. S. D. and EDELMAN J. (1951) The carbohydrates of the Jerusalem artichoke and other Compositae. *Biochem. J.* **48**, 114–126.

BERGMAN F. and DIKSTEIN S. (1954) Studies on uric acid and related compounds. I. Quantitative determination of uric acid in biological fluids. *J. biol. Chem.* **211**, 149–153.

BERRY H. K., SUTTON H. E., CAIN L., and BERRY J. S. (1951) Development of paper chromatography for use in the study of metabolic patterns. *In* Biochemical Institute Studies IV. *Univ. Texas publication no.* 5109, 22–25.

BOUSSINGAULT M. (1872) Sur une matière sucrée apparue sur les feuilles d'un tilleul. *C.R. Acad. Sci., Paris* **74**, 87–90.

COCKING E. C. and YEMM E. W. (1954) Estimation of amino acids by ninhydrin. *Biochem. J.* **58**, (1), xii.

CONWAY E. J. (1950) *Microdiffusion analysis and volumetric error*. Crosby Lockwood, London.

DELAUNAY H. (1931) L'excrétion azotée des invertébrés. *Biol. Rev.* **6**, 265–301.

DENISON F. W. and PHARES E. F. (1952) Rapid method for paper chromatography of organic acids. *Analyt. Chem.* **24**, 1628.

DENT C. E. (1947) The aminoaciduria in Fanconi syndrome. *Biochem. J.* **41**, 240–253.

DUSPIVA F. (1953) Der Kohlhydratumsatz in Verdauungstrakt der Rhynchoten, ein Beitrag zur Problem der stofflichen Wechselbeziehungen zwischen saugenden Insekten und ihren Wirtspflanzen. *Mitt. biol. Zent. Anst. Berl.* **75**, 82–88.

DUSPIVA F. (1954) Weitere Untersuchungen uber stoffwechselphysiologische Beziehungen zwischen Rhynchoten und ihren Wirtspflanzen. *Mitt. Biol. Bundesanst. Berlin* **80**, 155–162.

ERMIN R. (1950) Untersuchungen zur Honigtau- und Tannenhonigfrage in der Türkei. *Rev. Fac. Sci. Univ. Istanbul (B)* **15**, 185–224.

EVANS D. R. and DETHIER V. G. (1957) The regulation of taste thresholds for sugars in the blowfly. *J. Ins. Physiol.* **1**, 3–17.

FEIGL F. (1947) *Qualitative analysis by spot tests*. Elsevier.

FINK R. (1952) Morphologische und physiologische Untersuchungen an den intrazellularen Symbioten von *Pseudococcus citri* Risso. *Z. Morph. Ökol. Tiere* **41**, 78–146.

FORSYTH W. G. C. (1948) Colour reagents for the paper chromatography of sugars. *Nature, Lond.* **161**, 239–240.

FRY B. A. (1955) *The nitrogen metabolism of micro-organisms*. Methuen, London.

GALE E. F. (1951) *The chemical activities of bacteria*. Univ. Tutorial Press, London.

GATES M. (1947) The chemistry of the pteridines. *Chem. Rev.* **41**, 63–95.

GEINITZ B. (1930) Die Entstehung des Tannenhonigs. *Arch. Bienenk.* **11**.

VOL. 3 1959

GRAY H. E. and FRAENKEL G. (1953) Fructomaltose, a recently discovered trisaccharide isolated from honeydew. *Science* **118**, 304–305.

GRAY H. E. and FRAENKEL G. (1954) The carbohydrate components of honeydew. *Physiol. Zoöl.* **27**, 56–65.

GRAY R. A. (1952) Composition of honeydew excreted by pineapple mealybugs. *Science* **115**, 129–133.

HANES C. S. and ISHERWOOD F. A. (1949) Separation of the phosphoric esters on the filter paper chromatogram. *Nature, Lond.* **164**, 1107–1112.

HOUSE H. L. (1956) Nutritional requirements and artificial diets for insects. *Rep. ent. Soc. Ontario* **86**, 5–9.

JONES A. S., LEE W. A., and PEACOCKE A. R. (1951) The determination of phosphorus in deoxypentose nucleic acids. *J. chem. Soc.* **133**, 623–624.

KENNEDY J. S. and MITTLER T. E. (1953) A method of obtaining phloem sap via the mouth-parts of aphids. *Nature, Lond.* **171**, 528.

LAMB K. P. (1953) Aphid honeydew. *N.Z. Sci. Rev.* **11**, 86.

LAMB K. P. (1957) Quantitative micro-analysis of leaf tissue for certain organic constituents with special reference to sampling error. *N.Z. J. Sci. Tech. (B)* **38**, 1009–1017.

LINDEMANN C. (1948) Beitrag zur Ernährungsphysiologie der Blattläuse. *Z. vergl. Physiol.* **31**, 112–133.

LUGG J. W. H. and OVERELL B. T. (1947) Partition chromatography of organic acids on a paper sheet support. *Nature, Lond.* **160**, 87–88.

MALTAIS J. B. and AUCLAIR J. L. (1952) Occurrence of amino acids in the honeydew of the crescent-marked lily aphid, *Myzus circumflexus* (Buck.). *Canad. J. Zool.* **30**, 191–193.

MARKHAM R. (1942) A steam distillation apparatus suitable for micro Kjeldahl nitrogen determination. *Biochem. J.* **36**, 790.

MARKHAM R. and SMITH J. D. (1949) Chromatographic studies on nucleic acids. I. *Biochem. J.* **45**, 294–298.

MARKHAM R. and SMITH J. D. (1952) The structure of ribonucleic acids. I. *Biochem. J.* **52**, 552–557.

MICHEL E. (1942) Beiträge zur Kenntnis von *Lachnus* (*Pterochloris*) *roboris* L., einer wichtigen Honigtauerzeugerin. *Z. angew. Ent.* **29**, 243–281.

MITTLER T. E. (1953) Amino acids in phloem sap and their excretion by aphids. *Nature, Lond.* **172**, 207.

NELSON N. (1944) A photometric adaptation of the Somogyi method for the determination of glucose. *J. biol. Chem.* **153**, 375–380.

PALMER-JONES T., SUTHERLAND M. D., PATERSON C. R., HARRIS W. F., and FILMER D. W. (1947) A recent outbreak of honey poisoning. *N.Z. J. Sci. Tech. (A)* **29**, 107–143.

PARTRIDGE S. M. (1949) Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. *Nature, Lond.* **164**, 443.

PARTRIDGE S. M. and WESTALL R. G. (1948) Filter paper partition chromatography of sugars. *Biochem. J.* **42**, 238–250.

PIRT S. J. and WHELAN W. J. (1951) The determination of starch by acid hydrolysis. *J. Sci. Fd Agric.* **2**, 224–228.

PUCHER G. W., VICKERY H. B., and LEAVENWORTH C. S. (1935) Determination of ammonia and of amide nitrogen in plant tissue. *Industr. Engng Chem. (Anal. Ed.)* **7**, 152–156.

RUSSELL J. A. (1944) The colorimetric estimation of small amounts of ammonia by the phenol-hypochlorite reaction. *J. biol. Chem.* **156**, 457–461.

SMITH J. D. (1948) Symbiotic micro-organisms of aphids and fixation of atmospheric nitrogen. *Nature, Lond.* **162**, 930–931.

SNELL F. D. and SNELL C. T. (1953) *Colorimetric methods of analysis* **3**, 435. Van Nostrand.

SOMOGYI M. (1945) A new reagent for the determination of sugars. *J. biol. Chem.* **160**, 61–68.

TÓTH L. (1940) The protein metabolism of aphids. *Ann. Mus. Hist. nat. Hungary* **33**, 167–170.

TÓTH L., WOLSKY A., and BATYKA E. (1944) Stickstoffassimilation aus der Luft bei Rhynchoten (Insecta). *Z. vergl. Physiol.* **30**, 300–320.

TREVELYAN W. E., PROCTOR D. P., and HARRISON J. S. (1950) Detection of sugars on paper chromatograms. *Nature, Lond.* **166**, 444–445.

TRUSZKOWSKI R. and CHAJKINÓWNA S. (1935) Nitrogen metabolism of certain invertebrates. *Biochem. J.* **29**, 2361–2365.

VARNER J. E., BULLEN W. A., VANECKO S., and BURRELL R. C. (1953) Determination of ammonium, amide, nitrite and nitrate nitrogen in plant extracts. *Analyt. Chem.* **25**, 1528.

WATERHOUSE D. F. (1957) Digestion in insects. *Ann. Rev. Ent.* **2**, 1–18.

WEBER H. (1929) Aphidina. Blattläuse. *Biol. Tiere Deutschlands* **31**, 209–286.

WHITE J. W. and MAHER J. (1953) Transglycosidation by honey invertase. *Arch. Biochem. Biophys.* **42**, 360–367.

WIGGLESWORTH V. B. (1931) The physiology of excretion in a blood sucking insect. III. The mechanism of uric acid secretion. *J. exp. Biol.* **8**, 443–451.

WILSON P. W. and BURRIS R. H. (1947) The mechanism of biological nitrogen fixation. *Bact. Rev.* **11**, 41–73.

WOLF J. P. and EWART W. H. (1955) Carbohydrate composition of the honeydew of *Coccus hesperidum* L.: evidence for the existence of two new oligosaccharides. *Arch. Biochem. Biophys.* **58**, 365–372.

YEMM E. W. and WILLIS A. J. (1954) The estimation of carbohydrates in plant extracts by anthrone. *Biochem. J.* **57**, 508–514.

ZOEBELEIN G. (1956) Eie Honigtau als Nahrung der Insekten. *Z. angew. Ent.* **38**, 369–416; **39**, 129–167.

VOL.
3
1959

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 14 *to* 27. *Pergamon Press Ltd., London. Printed in Great Britain*

THE SYSTEMIC INSECTICIDAL ACTION OF PARAOXON ON THE EGGS OF *PIERIS BRASSICAE* (L.)

W. A. L. DAVID

Agricultural Research Council, Unit of Insect Physiology, Storey's Way, Cambridge

(*Received* 6 *May* 1958)

Abstract—When cabbage plants bearing eggs of *Pieris brassicae* L. were watered at the roots with paraoxon the eggs failed to hatch although the larvae within them developed apparently normally. The eggs were also killed when laid on turnip and mustard plants, and there was some effect on eggs laid on broad beans. The eggs of two other species of Lepidoptera were not affected.

The poison reached the eggs through the point of attachment to the leaves. No appreciable fumigant action occurred since eggs lying loosely on the leaves hatched normally. Paraoxon was demonstrated in the leaf wax and a material toxic to *Aedes aegypti* larvae was found in the fluid contents of the eggs on treated plants.

Cholinesterase activity could be detected in homogenates of the developing *Pieris* eggs after the second to third day. The level of activity was always lower in eggs from treated than from control plants, showing that an inhibitor was present in the eggs from treated plants.

A material active on muscle preparations was detected in the eggs after the fourth day. This was shown to be ACh and more was always present in eggs from treated than from control plants. The concentration difference was greatest at the time the eggs were due to hatch. The evidence suggests that ultimately it was the high ACh content of eggs from treated plants that was responsible for their failure to hatch.

INTRODUCTION

It has already been reported that when solutions of paraoxon and demeton were watered on to the compost in which young cabbage plants were growing, eggs of *Pieris brassicae* L., the large white butterfly, on the leaves were prevented from hatching although the larvae within them apparently developed normally (David and Gardiner, 1953, 1954). In fact, in many cases, the larva nibbled a hole in the shell at the side of the egg near the top but got no further.

A systemic ovicidal action of this kind does not appear to have been reported previously. It has however been noted that some effective ovicides did not stop development but prevented the mature larvae from emerging (Potter and Tattersfield, 1943; Salkeld and Potter, 1953; Smith and Avens, 1954).

This paper describes experiments which have been carried out to follow the transfer of toxic material from plants treated with paraoxon to the eggs. The effect on the acetylcholine content and cholinesterase activity of the eggs has also been determined.

MATERIALS AND METHODS

The sample of paraoxon, diethyl *p*-nitrophenyl phosphate, used in these experiments was highly purified. Further information about its properties has been given by David and Gardiner (1951).

The *Pieris* culture and the cabbage plants were kept, and the experiments involving growing plants were carried out, in the greenhouse previously described (DAVID and GARDINER, 1951).

In the experiments with paraoxon the insecticide was applied by pouring the required dose, in 20 ml of solution, on to the soil in which the cabbage plants bearing *Pieris* eggs were growing. In this way the toxic material only reached the eggs systemically after passing through the plants to the point of attachment of the eggs. Tests showed that under these conditions a fumigant action from the material on the soil did not occur.

METHODS FOR OBTAINING AND HANDLING THE EGGS

The *Pieris* eggs were obtained from a laboratory culture maintained all the year round (DAVID and GARDINER, 1952; DAVID, 1957). When the eggs were to be used for the biochemical work they were collected only between 10 a.m. and 2 p.m. so as to restrict the age range. The young cabbage plants bearing the eggs were kept in a constant temperature cabinet at 20°C±1°C in the shaded part of a cooler greenhouse.

VOL. 3 1959

For many of the experiments it was necessary to detach large numbers of eggs from the leaves. Although it is quite possible to do this without damaging the eggs by pushing them gently with a fine sable brush, it is much quicker to use acetone to soften the wax and cement (BEAMENT and LAL, 1957). In the present experiments the leaves were dipped momentarily in acetone, the excess was shaken off and the eggs were removed into a porcelain evaporating dish with the fine brush. After this treatment all the acetone must be evaporated before the eggs are weighed. Observation showed that the treated eggs hatched normally.

DETERMINATION OF CHOLINESTERASE

The cholinesterase activity of the eggs was measured at 37°C and at *p*H 7·4 in bicarbonate buffer (0·023 M–$NaHCO_3$ in equilibrium with 5 per cent v/v CO_2 in N_2 in the gas phase) using the Warburg manometric technique as modified by AMMON (1933). Acid formed during the hydrolysis of the ester substrate released CO_2 from the buffer, and the rate of hydrolysis was measured as μl CO_2/g eggs/15 min. For each Warburg flask, 0·1 to 0·2 g of eggs was allowed. They were homogenized in 0·023 M–$NaHCO_3$ at 0–2°C in an all-glass homogenizer since, as would be expected, whole eggs did not show any cholinesterase activity. Glass distilled water was used for making up the bicarbonate solution. The final volume was adjusted so that the weight of eggs per ml fell within the above mentioned range.

Several substrate concentrations from 1 to 15 mM were tested, and a concentration of 2·67 mM acetylcholine (ACh) bromide in the final solution was found to be satisfactory.

Bio-assay of rectus stimulating material shown to be acetylcholine (ACh)

The isolated rectus abdominis muscle of the frog was used for most ACh assays (CHANG and GADDUM, 1933). In certain cases the assay was repeated on

isolated guinea-pig ileum (cf. BANISTER, WHITTAKER, and WIJESUNDERA, 1953). As will be described later, tests were made to show that the active material was in fact ACh and that no activating or inhibiting substances were present in the extracts (FELDBERG and HEBB, 1947).

The extract for these tests was prepared by homogenizing the eggs (about 0·5 g) in 2 ml cold 10 per cent trichloroacetic acid (TCA) (cf. MACINTOSH and PERRY, 1950) in the cold room as before. The homogenate was centrifuged in the cold at 11,000 rev/min in a Serval centrifuge, the precipitate re-extracted, and the extract and washings (after re-centrifuging) transferred to a graduated centrifuge tube and extracted with ether to remove TCA and fat. Ether extraction was continued until the solution reached 4·5 *p*H (bromocresol green as an external indicator) and the ether was removed by an air jet. The solution so obtained was used for the assays. The unit of activity employed was the amount of synthetic ACh perchlorate in mμ moles giving an equal response on the frog rectus or guinea-pig ileum when tested in a concentration range over which the dose response curve of the preparation is approximately linear (GARDINER and WHITTAKER, 1954).

Chromatography on columns of ion-exchange resin

To obtain sufficient rectus-stimulating material for column chromatography, extracts were made from eggs laid on treated plants on several successive days. The eggs were homogenized just before they were due to hatch when the larvae within them appeared to be fully developed, since the content of active material was highest at this time.

The extracts, prepared as described for the bio-assay of ACh, were assayed individually, then pooled and concentrated to about 1·0 ml by evaporation at 30–40°C under reduced pressure before transferring to the column.

The column used was packed with a bed of specially prepared ion-exchange resin, Amberlite XE-97, 12 cm long and 0·6 cm in diameter (GARDINER and WHITTAKER, 1954). The column was run at room temperature (20–25°C) with 0·1 M–NaH_2PO_4 as the eluting agent at a flow rate of 18 ml/hr and fractions of constant volume were collected with an automatic fraction collector.

These fractions were assayed using the frog rectus abdominis muscle.

Later the most active fractions were pooled, evaporated to dryness and the active material was extracted from the phosphate buffer by three washes of 2 ml of absolute alcohol. The alcohol was evaporated and the residue from each extraction was taken up in 1 ml water and assayed in the usual way. The same column was also run with a known quantity of synthetic ACh for comparison.

RESULTS

Dipping tests with ova

Tests in which the leaves of cabbage plants bearing *Pieris* eggs were dipped in solutions of paraoxon (E 600) have already been reported (DAVID and GARDINER,

1953). The eggs tested were all within 48 hr of hatching. In the present experiments eggs of two more narrowly restricted age ranges have been used and an additional factor has been taken into account. This is whether, after dipping, the dose which the developing larva receives comes only from the egg shell or whether paraoxon also diffuses in from the leaf wax as it will be shown to do when the plants are treated systemically via the roots.

Experiments have been carried out in which whole cabbage plants bearing *Pieris* eggs on one leaf have been dipped in solutions of paraoxon at 18–20°C containing 0·1 per cent "Teepol" as a wetting agent. The plants were dried on their sides and immediately afterwards some of the eggs were removed to watch glasses with a camel-hair brush. This was done with eggs less than 4 hr old and also with eggs within 24 hr of hatching. This procedure was intended to ensure that the deposit on the eggs removed from the leaves was the same as that on the eggs left on the plant. The results are shown in Table 1 and it can be seen that eggs dipped when less than 4 hr old were more resistant to paraoxon than eggs dipped within 24 hr of hatching and that eggs removed from the plant were less affected than eggs left on the plant.

VOL. 3 1959

TABLE 1—*Pieris* EGGS DIPPED IN PARAOXON SOLUTIONS AND EITHER LEFT ON THE PLANT OR REMOVED AFTER DRYING

Concentration %	Percentage of larvae killed within the shells	
	Eggs on plants	Eggs removed from plants
	Eggs less than 4 hr old	
0·005	99	100
0·001	70	21
0·0005	3	7
0·0001	0	0
	Eggs within 24 hr of hatching	
0·005	100	100
0·001	87	49
0·0005	52	10
0·0001	0	0
Control (both ages)	0	0

Each figure is the average of 6 determinations

The duration of the toxic effect

When testing for a systemic effect against the eggs the paraoxon solutions might have been poured on to the soil in which the cabbage plants were growing either before or after the eggs were laid. The influence of different timing procedures was investigated to determine when the toxic action was most pronounced.

The cabbage plants were growing in 3 in. diameter pots. The paraoxon was applied in 20 ml of solution at either 0·02 per cent or 0·01 per cent. The eggs were laid on all the plants on the same day. Some of the plants had already been

treated and others were treated subsequently as indicated in Table 2. Eggs which hatched did so on the sixth day.

All the results agree in showing that the maximum effect on the eggs occurred when the insecticide was applied on the same day as the eggs were laid or a day or two afterwards.

Table 2—Cabbage plants treated by pouring paraoxon solutions on the soil in which they were growing before or after *Pieris* eggs were laid on them

Plant treated before or after eggs laid	Percentage of larvae killed within the shells		
	0·02% paraoxon		0·01% paraoxon
	Exp. 1	Exp. 2	Average of 3 Experiments
10 days before	0	0	0
5 days before	32	40	2
2 days before	45	95	58
On the same day	99	100	73
2 days after	90	95	75
4 days after	99	80	73
Control eggs on untreated plant	0	0	0

The systemic action against eggs on other plants

Experiments were carried out to determine whether *Pieris* eggs laid on plants other than cabbages were also prevented from hatching by paraoxon applied systemically. This question is of interest since movement of the insecticide into the egg is likely to be influenced by the surface structure of the leaf.

It was found that *Pieris* eggs laid on turnip and mustard plants were prevented from hatching just as they were on cabbages. A dose of 2 mg paraoxon in 20 ml solution poured on to the standard 3 in. diameter pot in which the plant was growing 24 hr after the eggs had been laid prevented 95–100 per cent from hatching. *Pieris* were also induced to lay on broad bean plants. In this case paraoxon was less effective: 20 mg applied under the same circumstances only prevented about 50 per cent of the eggs from hatching. All the eggs on control plants hatched normally.

It is clear that the effect is in no way peculiar to cabbage plants.

The systemic action of paraoxon against the eggs of other Lepidoptera

All the eggs of *Diataraxia oleracea* L., the tomato moth, hatched on the leaves of a cabbage plant growing in the standard pot watered with 20 cc of 0·02 per cent paraoxon solution (4·0 mg/pot). The eggs were rather loosely attached to the plant and some were laid on top of others. *Pieris* eggs on the same plant did not hatch.

In a similar experiment with eggs of *Plutella maculipennis* Curt., the diamond-back moth, over 95 per cent hatched. These eggs are not so precisely orientated on the plant as those of *Pieris* and are not so firmly cemented. It was noted, however, that when they were pushed off the leaf some wax was detached as with *Pieris*. Again *Pieris* eggs on the same plant did not hatch. Also, unlike the *Pieris* larvae, the young *Plutella* larvae survived on the treated plant indicating that they were comparatively resistant to paraoxon.

The transfer of toxic material to the eggs

The attachment of the eggs to the leaf. *Pieris* eggs are ribbed longitudinally except at the basal (posterior) portions. This is smooth and flattened against the leaf. At the time of laying the female covers the surface of the egg with a cement which fixes it to the leaf surface. The cement consists of protein and an unsaturated lipoid not attached chemically to the protein. (BEAMENT and LAL, 1957.) When it comes into contact with the waxy surface of the cabbage leaf the wax appears to be softened and a strong adhesion is formed. So strong is this adhesion that if the egg is pushed off the leaf the underlying wax is completely stripped with it leaving the leaf cuticle exposed. It may be assumed that the eggs rest on a mixed layer of cement and wax. Round the base of the egg there is a shoulder of excess cement which makes the attachment firmer.

The importance of the cemented attachment. The *Pieris* eggs on cabbage plants watered at the roots with paraoxon could theoretically be killed either by a fumigant action or by toxic material transferred to the eggs through the point of attachment.

TABLE 3—*Pieris* EGGS ON THE LEAVES OF CABBAGE PLANTS WATERED AT THE ROOTS WITH 20 ML OF 0·1 PER CENT PARAOXON AND ON UNTREATED PLANTS

Treatment of eggs and position on leaf	Number of eggs hatching out of total used
On treated plants	
Normally attached to the upper surface	8/166
Normally attached to the lower surface	3/250
Loose on the upper surface	9/9
Loose on the lower surface	10/10
Stuck with cement to the upper surface	2/10
Stuck with cement to the lower surface	0/5
On control plants	
Normally attached to the upper surface	149/150
Loose on the upper surface	10/10
Stuck with cement to the upper surface	13/14

However, if newly laid eggs detached from untreated plants were placed loosely on the upper or under surface of the leaves of treated plants and left there they were not prevented from hatching while eggs naturally attached to the same leaves were. This showed that the lethal effect was not brought about by fumigant

action and incidentally that eggs in loose contact with the leaves were not affected. If the detached eggs were stuck to the leaves of treated cabbage plants with drops of cement taken from the cement glands of a female *Pieris* they were prevented from hatching. The toxic material must therefore pass into the egg through the base where it is cemented on to the leaf (Table 3).

Transfer of toxic material from the plant to the eggs. Since it was only eggs which were cemented to the surface of the leaf of a treated plant that were affected, it could be suggested that a solvent action of the cement on the leaf wax had allowed the base of the egg to come into direct contact with the cuticle of the leaf and that this contact was essential for the transference of the insecticide. This suggestion has been disproved by the observation that eggs laid on thin films of cabbage wax containing 0·05 per cent paraoxon on microscope cover glasses also did not hatch. Here again a fumigant effect was excluded by having some *Pieris* eggs on narrow strips of thin cover slip lying on the wax film beside the eggs laid directly on it. These eggs hatched normally.

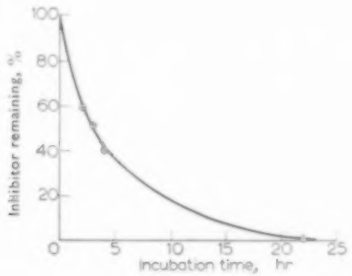


Fig. 1. The concentration of inhibitor, expressed as a percentage of the initial concentration, was estimated by means of cholinesterase inhibition measurements and a standard curve determined with paraoxon, relating inhibition to inhibitor concentration. + = Hydrolysis of paraoxon ; ○ = Hydrolysis of inhibitor in extract.

The alternative suggestion, that the toxic material passes into the egg from the wax on the surface of the leaf, in fact receives support from this experiment. But further proof depends on toxic material being demonstrated in the wax on the leaves of treated plants.

Two cabbage plants were treated in the usual way by pouring 20 ml of 0·1 per cent paraoxon solution on to the soil and 5 days later the leaves were picked and the wax washed off by dipping them momentarily in chloroform. Leaves from control plants were treated similarly. The chloroform extracts were filtered and the chloroform was removed by distillation and finally by a stream of compressed air. The plant wax so obtained was then extracted with hot water and the extract was filtered. First instar larvae of *Aedes aegypti* L. were used to detect any toxic material which might be present in these extracts. All the larvae were killed by extracts from the treated plants even when it was diluted to 50 per cent, whereas extracts of control plants had no effect on the larvae.

The nature of the toxic material in the leaf wax. Some of the active extract was examined by Dr. W. N. Aldridge using the methods previously referred to (David and Aldridge, 1957). It was found that the extract was a very active cholinesterase inhibitor, only 0·046 μl of extract/ml of washed sheep erythrocyte suspension being required to produce 50 per cent inhibition in 30 min at 37°C compared with more than 1100 μl/ml for the extract from the control plants.

When the rate of hydrolysis of the inhibitor was compared with that of paraoxon under the same conditions as previously described (David and Aldridge, 1957) it was found that they were identical (Fig. 1).

It may be concluded that the paraoxon applied to the soil reaches the wax on the surface of the leaves.

The occurrence of toxic material within the eggs. In the introduction it was mentioned that very often the larvae began to cut their way out of the shells but failed to emerge. It might be thought therefore that the toxic material was confined to the shells and that larvae only acquired a toxic dose when they began to gnaw into the various layers of the walls. This is not so. In the first place, in some experiments, the larvae nearly all died without visibly puncturing the shells and even the suggestion that the poison was confined to some inner layer can also be convincingly disproved by the demonstration of toxic material in the fluid surrounding the developing larvae. This does not mean however that additional toxic material was not taken in by the larvae when they ate the holes in the shells.

Ten eggs which were about due to hatch on treated plants were punctured and the fluid contents were withdrawn into a fine capillary. The fluid was diluted with a drop of water containing a first stage mosquito larva held in a slightly wider capillary tube. Control tests were carried out with fluid taken from 10 eggs on an untreated cabbage plant and with larvae in water. The larvae in the extract from the eggs on the control plant diluted 1 : 3 and in water were apparently normal after 18 hr in the capillary tubes, whereas the larvae in the extract from the eggs on the treated plants diluted 1 : 3 and 1 : 6 were motionless and apparently dead after 2 hr. It is clear therefore that the eggs on the treated plants contain some substance toxic to mosquito larvae which is absent from the eggs on the control plants.

A further proof that the larvae were already affected within the shells before they began to gnaw their way out was obtained by dissecting the larvae out of the shells about 6 hr before they were due to hatch. Larvae removed at this stage and placed on small pieces of cabbage leaf in a moist atmosphere remained almost motionless for some hours. Later those from control plants became active and fed but those taken from eggs on plants treated systemically with paraoxon shrivelled and died.

The claim that the toxic material acts upon the larvae within the shells is also supported by the results given in the following section.

The cholinesterase content of Pieris *eggs from treated and control plants*

After a preliminary experiment to determine the approximate cholinesterase activity of homogenates of eggs of various ages a comparative experiment with

eggs from treated and control plants was arranged. Twenty ml of 0·1 per cent paraoxon solution was poured on to the soil in each of two 3-in. diameter pots in which cabbage plants were growing. These plants were maintained in the constant temperature cabinet at 20°C overnight and next day they were placed in the adult *Pieris* cage with 2 control plants so that eggs could be laid on them. They were then returned to the cabinet. This procedure was repeated with other plants for 5 days and on the sixth day all the eggs were removed, homogenized and used for the cholinesterase determination as described. The results are given in Fig. 2. Very little cholinesterase was detected in the eggs less than 3 days old

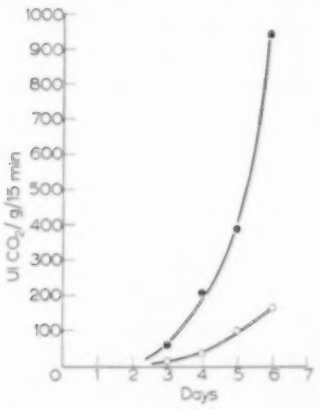


Fig. 2. The cholinesterase activity, expressed as $\mu l \cdot CO_2/g$ eggs/15 min., of *Pieris* eggs of different ages taken from plants treated with paraoxon via the roots and from control plants. ○ = Eggs from plants treated with paraoxon ; ● = Eggs from control plants.

(at 20°C). After that it increased rapidly in the control eggs until hatching time was reached on the sixth day. The eggs from treated plants always showed much less activity and the difference was very striking by the time the eggs were due to hatch.

The acetylcholine in eggs from treated and control plants

It will be shown later that the rectus-stimulating material from the eggs is ACh and it is convenient to anticipate this result in reporting the present experiment.

As the cholinesterase in eggs from treated plants was inhibited it was also expected that their ACh content would be high compared with eggs from control plants. Young potted cabbage plants were treated and the *Pieris* eggs were collected as for the cholinesterase determination. The ACh extraction was carried out as described under 'Methods'.

The results of two comparative experiments are shown in Table 4. Very little ACh can be detected in the eggs before the fifth day. Both experiments

show, however, that the ACh content of the eggs on the treated plants, which failed to hatch, was consistently higher than that of eggs which hatched and that the difference was greatest at the time of hatching.

TABLE 4—THE EFFECT OF PARAOXON APPLIED SYSTEMICALLY TO THE ROOTS OF CABBAGE PLANTS ON THE ACh CONTENT OF THE EGGS OF *Pieris brassicae* LAID ON THE LEAVES

Age of eggs (days at 20°C approximately)	First experiment Eggs deposited on:		Second experiment Eggs deposited on:	
	Treated plants	Control plants	Treated plants	Control plants
	μg ACh chloride g eggs		μg ACh chloride g eggs	
1	0	0	—	—
2	0	0	—	—
3	0	0	—	—
4	0·6	0	—	—
5	8·5	0·9	20·6	7·6
6	31·5	13·7	22·9	9·6
7	—	—	76·0	9·8*

* These eggs had just hatched and the young larvae were used for the assay

Confirmation that the rectus stimulating material is acetylcholine

To obtain as much active material as possible for these experiments eggs on plants dosed with 0·1 per cent paraoxon via the soil were used when the larvae were about due to hatch. Four batches of eggs were collected. These weighed altogether 5·74 g and the extract prepared from them had a volume of 22·6 ml. It was shown by assay to contain the equivalent of about 127 μg of ACh chloride or 698 mμ moles.

This active material extracted from egg homogenates was chromatographed on specially prepared Amberlite resin XE-97 as described. Eighty 2 ml fractions were collected and the distribution of the rectus stimulating material in the effluent was determined in comparison with synthetic ACh. All the activity centred around one peak occurring approximately in the position expected for ACh. The recovery was 77 per cent. The active material was shown to be alkali labile and its activity on the rectus was potentiated by eserine.

When the assays were repeated on the ileum, again using synthetic ACh as a standard, essentially the same result was obtained. Since the relative potency of other esters on these two test systems is in general widely different from that of ACh the identity of the rectus and ileum results in the present instance is excellent evidence that the active substance is, in fact, ACh. The recovery of activity was 78 per cent (Fig. 3). The ileum activity was not inhibited by the antihistamine N-*p*-methoxybenzyl-N-N-dimethyl-N-α-pyridylethylene-diamine maleate (1 μg/ml) and was completely inhibited with atropine (0·2 μg/ml).

All the effluent fractions containing active material were pooled and after concentrating and extracting from the phosphate the active material was again

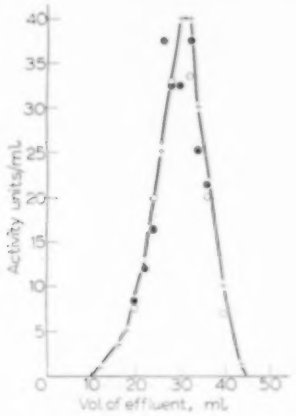


FIG. 3. Chromatography of concentrate of extract from *Pieris* eggs, taken from plants treated with paraoxon, on XE-97. For definition of activity units and other details see text. ● = First assay on rectus ; ○ = Second assay on rectus ; + = Assay on ileum.

assayed. About 34 per cent of the quantity calculated to be present in the pooled effluent fractions was recovered, equivalent to about 147 mμ moles of ACh.

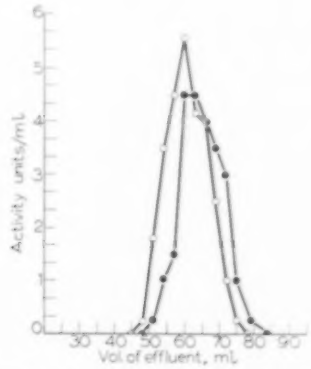


FIG. 4. Chromatography of the concentrated effluent fractions containing active material obtained from the first chromatograph (Fig. 3) run in comparison with a similar amount of synthetic ACh on a fresh column containing XE-97. For definition of activity units and other details see text. ○ = Extract from eggs ; ● = Control acetylcholine perchlorate.

A fresh XE-97 column was prepared and 100 mμ moles of synthetic ACh was run on it followed by 106 mμ moles of the active material from the eggs. In these cases 3 ml fractions were collected. The distribution of activity of the 2 samples

in the effluent fractions as assayed on the rectus was almost identical. The recovery of the control synthetic ACh was 72 per cent and of the unknown 78 per cent (Fig. 4). The fraction of the unknown which had the maximum activity was assayed on the ileum also and gave results in exact agreement with the rectus assay.

It may be concluded that the active material extracted from the eggs is ACh.

DISCUSSION

It is known from earlier work that organic phosphorus compounds can act as contact ovicides. SALKELD and POTTER (1953) worked with *Diataraxia oleracea* L., *Ephestia kühniella* Zell., and *Dysdercus fasciatus* Sign. They showed that comparatively low dosages of HETP gave a 100 per cent kill but that much higher dosages were required to inhibit development. SMITH and AVENS (1954) have shown that parathion will give a 100 per cent kill of the eggs of *Sanninoidea exitiosa* Say, but in this case also the embryos developed apparently normally to the point of hatching. The similar effect as a result of the systemic action of paraoxon and demeton on *Pieris* eggs discussed here has already been mentioned (DAVID and GARDINER, 1953, 1954).

VOL. 3 1959

It is not surprising that *Pieris* embryos are killed by paraoxon since the various shell and embryonic layers are relatively permeable to oil soluble poisons (BEAMENT and LAL, 1957). But the fact that at concentrations sufficient to give 100 per cent kill death does not occur in this and other cases until development is complete has led to speculation as to whether the organic phosphorus poison only reaches the embryo at some late stage in development when membranes are being broken down just prior to hatching, or whether it penetrates but is without effect on the very young embryo (STAUDENMAYER, 1953; POTTER, LORD, KENTEN, SALKELD, and HOLBROOK, 1957). As pointed out earlier this delayed action occurs with many other insecticides and is not peculiar to organic phosphorus compounds (POTTER and TATTERSFIELD, 1947; SALKELD and POTTER, 1953; SMITH and AVENS, 1954; SPEYER, 1950).

There is now sufficient evidence to discount the suggestion that the organic phosphorus compounds are accumulated by the layers external to the embryo and prevented from reaching it until these layers are broken down. SMITH (1955) has shown that treatment with parathion depressed the respiratory rate of peach tree borer eggs during the last 2 days before hatching was due (which normally took $8\frac{1}{2}$ days) and STAUDENMEYER (1957) has shown an earlier effect of paraoxon on silkworm eggs. Furthermore, since it is well known that organic phosphorus compounds are active anticholinesterases, in seeking to explain their action it was natural to look for some effect on this system. STAUDENMAYER (1955) found that cholinesterase in silkworm eggs was inhibited by parathion and paraoxon. SMITH and WAGENKNECHT (1956) showed that parathion penetrated the eggs of the peach tree borer (*Sanninoidea exitiosa* Say) and inhibited cholinesterase as soon as it appeared. MEHROTRA and SMALLMAN (1957) found that parathion completely inhibited the cholinesterase activity of eggs of the house-fly and greatly increased

the ACh content. These observations show conclusively that the poison has reached the larvae.

The present work on *Pieris* eggs treated with paraoxon systemically through the cabbage plants confirms these findings. The cholinesterase activity of eggs from treated plants was appreciably lower than that of eggs from control plants at all times after cholinesterase could be detected. It could be argued that this inhibition only occurred when the paraoxon came into contact with the tissues of the embryo after the eggs had been homogenized. However, this suggestion was discounted by the observation that eggs from treated plants also contained comparatively much more ACh which could not have been synthesized after homogenization in TCA. It is reasonable to suppose that the paraoxon penetrated to the embryo, inhibited the cholinesterase and thereby led to the accumulation of ACh. The fact that toxic material was demonstrated in the fluid contents of the eggs adds further support to the view that it also reached the embryo.

That these changes occur in the developing, treated, eggs does not preclude the possibility that at the time of hatching the larvae are exposed to further dosages of poison. At this time the epembryonic membranes are broken down and the larvae imbibe the amniotic fluid and begin to eat through the shells. Since the poison must penetrate through the shells and is known to occur in the fluid content of the eggs the larvae must in fact be exposed to an additional dose at this time. But whether or not it contributes significantly to the poisoning of the larvae is not known.

Acknowledgements—The determinations of cholinesterase activity and ACh in egg homogenates were carried out in the Department of Biochemistry of the A.R.C. Institute of Animal Physiology at Babraham. I should like to thank Sir Rudolph Peters for kindly allowing me to work in his Department and particularly Dr. V. P. Whittaker who gave constant advice and in whose laboratory the work was carried out.

I should also like to thank here Dr. W. N. Aldridge who collaborated in an earlier part of the work as acknowledged in the text, Mr. B. O. C. Gardiner who maintained the *Pieris* culture which produced the large number of eggs required and Miss J. M. Gilson for technical assistance.

REFERENCES

Ammon R. (1937) Die fermentative spaltung des Acetylcholins. *Pflüg. Arch. ges. Physiol.* **233**, 486–491.

Banister R. J., Whittaker V. P., and Wijesundera S. (1953) The occurrence of homologues of acetylcholine in ox spleen. *J. Physiol.* **121**, 55–71.

Beament J. W. L. and Lal R. (1957) Penetration through the egg-shell of *Pieris brassicae* L. *Bull. ent. Res.* **48**, 109–125.

Chang C. H. and Gaddum H. J. (1933) Choline esters in tissue extracts. *J. Physiol.* **79**, 255–285.

David W. A. L. (1957) Breeding *Pieris brassicae* L. and *Apanteles glomeratus* L. as experimental insects. *Z. Pflkrankh.* **64**, 572–577.

David W. A. L. and Aldridge W. N. (1957) The insecticidal material in leaves of plants growing in soil treated with parathion. *Ann. appl. Biol.* **45**, 332–346.

DAVID W. A. L. and GARDINER B. O. C. (1951) Investigations on the systemic insecticidal action of sodium fluoroacetate and of three phosphorus compounds on *Aphis fabae* Scop. *Ann. appl. Biol.* **38**, 91–110.

DAVID W. A. L. and GARDINER B. O. C. (1952) Laboratory breeding of *Pieris brassicae* L. and *Apanteles glomeratus* L. *Proc. R. ent. Soc. Lond.* (A) **27**, 54–56.

DAVID W. A. L. and GARDINER B. O. C. (1953) The systemic insecticidal action of sodium fluoroacetate and of three phosphorus compounds on the eggs and larvae of *Pieris brassicae* L. *Ann. appl. Biol.* **40**, 403–417.

DAVID W. A. L. and GARDINER B. O. C. (1954) The action of the systemic insecticide systox on *Pieris brassicae* L. and *Phaedon cochleariae* F. *Bull. ent. Res.* **45**, 693–702.

FELDBERG W. and HEBB C. (1947) The effects of magnesium ions and of creatine phosphate on the synthesis of acetylcholine. *J. Physiol.* **106**, 8–17.

GARDINER J. E. and WHITTAKER V. P. (1954) The identification of propionylcholine as a constituent of ox spleen. *Biochem. J.* **58**, 24–29.

MACINTOSH F. C. and PERRY W. L. M. (1950) *Biological estimation of acetylcholine. Methods in Medical Research* **3**, 78–92 (Chicago Year Book, 1950).

MEHROTRA K. N. and SMALLMAN B. N. (1957) Ovicidal action of organo-phosphorus insecticides. *Nature, Lond.* **180**, 97–98.

POTTER C., LORD K. A., KENTEN J., SALKELD E. H., and HOLBROOK D. V. (1957) Embryonic development and esterase activity of eggs of *Pieris brassicae* in relation to TEPP poisoning. *Ann. appl. Biol.* **45**, 361–375.

POTTER C. and TATTERSFIELD F. (1943) Ovicidal properties of certain insecticides of plant origin (nicotine, pyrethrins, derris products). *Bull. ent. Res.* **34**, 225–244.

SALKELD H. and POTTER C. (1953) The effect of age and stage of development of insect eggs on their resistance to insecticides. *Bull. ent. Res.* **44**, 527–580.

SMITH E. H. (1955) Further studies on the ovicidal action of parathion to eggs of the peach tree borer. *J. econ. Ent.* **48**, 727–731.

SMITH E. H. and AVENS A. W. (1954) The ovicidal action of parathion in control of the peach tree borer. *J. econ. Ent.* **47**, 912–917.

SMITH E. H. and WAGENKNECHT A. C. (1956) The occurrence of cholinesterase in eggs of the peach tree borer and large milk weed bug and its relation to the ovicidal action of parathion. *J. econ. Ent.* **49**, 777–783.

SPEYER W. (1950) Haben die modernen Kontaktigifte eine ovicide Wirkung? Nachrichtenbl. *Dtsch. Pflanzenschutzd.* Jg. **2**, 2–3.

STAUDENMAYER T. (1953) The influence of "E 605" on the respiration of silkworm eggs. *Höfchenbr. Wiss.* (Engl. Edn.) **6**, 158–166.

STAUDENMAYER T. (1955) Die Cholinesterase während der Eientwicklung von *Bombyx mori* und die ovizide Wirkung von Phosphorsäureester (E600 u. E605). *Z. vergl. Physiol.* **37**, 416–423.

STAUDENMAYER T. (1957) Die Wirkung verschiedener Kontaktinsektizide auf die Atmung von Siederspinnereiern. *Z. vergl. Physiol.* **39**, 262–273.

VOL. 3 1959

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 28 *to* 40. *Pergamon Press Ltd., London. Printed in Great Britain*

HORMONAL STUDIES ON THE DIAPAUSE AND NON-DIAPAUSE EGGS OF THE SILKWORM, *BOMBYX MORI* L.

SEIJIRO MOROHOSHI

Gunze Silkworm and Mulberry Research Institute, Ayabe, Kyoto, Japan

(*Received* 10 *May* 1958)

Abstract—It is known that the brain-suboesophageal ganglion complex in the silkworm converts the non-diapause egg into the diapause egg. It has been found that the corpus allatum is concerned not only with the number of moults but also with the production of non-diapause eggs. The influence of the suboesophageal ganglion differs with each voltine race, and it gradually becomes stronger with age, whereas the influence of the corpus allatum differs with each moulting race and becomes weaker with age. The brain does not perfectly control the secretion of both the suboesophageal ganglion and the corpora allata, but weakly inhibits and also weakly promotes their secretory activity. The corpus allatum and suboesophageal ganglion hormones consist not only of non-diapause and diapause hormones, but also of growth-promoting and inhibitory hormones.

VOL
3
195

IT was independently verified by HASEGAWA (1951, 1952) and FUKUDA (1951, 1952) that voltinism in the silkworm depended on the secretion of the suboesophageal ganglion: HASEGAWA claimed that the voltinism can be determined by the suboesophageal ganglion alone, whereas FUKUDA assumed that it may be determined by the function of the brain-suboesophageal ganglion complex in the pupal stage. According to FUKUDA'S experiments, the brain absolutely controls the secretion of the suboesophageal ganglion. When the brain completely suppresses the secretion of the suboesophageal ganglion, moths lay non-diapause egg batches, and consequently he has concluded that the centre of the determination of voltinism exists in the pupal brain itself.

The present author and his collaborators planned certain experiments to examine these two different hypotheses concerning the determination of voltinism in the silkworm. Furthermore, they investigated the existence or non-existence of a hormone which converts the diapause egg into the non-diapause egg.

MATERIAL AND METHODS

Daizō race (bivoltine) and Cambodge race (multivoltine) were used as hosts in our transplantation experiments. When Daizō race is incubated at 15°C in the dark the moths always lay non-diapause egg batches. On the other hand, when incubated at 24°C in the light they always produce "diapause" moths. Several races were employed as donors in this experiment.

Transplantation experiments with the corpus allatum or the suboesophageal ganglion were performed in various stages of the fifth instar larva: more than

10,000 larvae in all were operated upon in 1956–57. The following descriptions are concerned only with typical results of some of the experiments.

The effects of the corpora allata and the brain-corpora allata complex on the production of non-diapause eggs could not be found in univoltine hosts. The effects were well observed only in Daizō and Cambodge hosts laying diapause eggs mixed with non-diapause ones.

EXPERIMENTAL RESULTS

(I) *Production of diapause eggs by transplanting the suboesophageal ganglion and the brain-suboesophageal ganglion complex*

(a) *Influence of larval age on the function of the transplanted suboesophageal ganglion.* When the suboesophageal ganglion and the brain-suboesophageal ganglion complex extracted from the "non-diapause" or "diapause" donors of Daizō race are transplanted into "non-diapause" hosts of the same race, some "non-diapause" hosts lay either diapause egg batches or egg batches mixed with diapause and non-diapause eggs, as seen in nos. 1–5 of Table 1 (see Figs. 4–6).

The percentage of diapause eggs produced increased with the age of the fifth instar larva when the suboesophageal ganglion or the brain-suboesophageal ganglion complex were transplanted.

TABLE 1—PRODUCTION OF DIAPAUSE EGGS BY TRANSPLANTATION OF BOTH THE SUBOESOPHAGEAL GANGLION (SG) AND THE BRAIN-SUBOESOPHAGEAL GANGLION COMPLEX (Br+SG)

No.	Recipient (Daizō race)	Donor	(Race)	Transplanted on 1/V			Transplanted on 3/V			Transplanted on 5/V		
				Number of			Number of			Number of		
				Non-diapause moths	Moths laid mixed eggs	Diapause moths	Non-diapause moths	Moths laid mixed eggs	Diapause moths	Non-diapause moths	Moths laid mixed eggs	Diapause moths
1.	Control	(Wounded)		39	0	0	36	0	0	43	0	0
2.	L ←—	—H(SG)		38	7	0	28	13	1	27	8	2
3.	L ←—	—H(Br+SG)	Di	43	8	0	27	16	2	21	9	12
4.	L ←—	—L(SG)		39	8	0	38	11	0	42	12	2
5.	L ←—	—L(Br+SG)		37	5	0	33	7	0	38	10	0
6.	L ←—	—H(SG)		—	—	—	7	18	14	—	—	—
7.	L ←—	—H(Br+SG)	E.16	—	—	—	4	13	35	—	—	—

Note: L, low incubation at 15°C; H, high incubation at 24°C; 1/V, 3/V, 5/V, transplantation on the first, third and fifth day of the fifth instar, respectively; Di, Daizō race; E.16, European no. 16.

(b) *Effect of brain taken from the "diapause" donor on the suboesophageal ganglion hormone in the "non-diapause" host.* The "non-diapause" hosts transplanted with the brain-suboesophageal ganglion complex from the "diapause" donor, as seen in nos. 2 and 3 of Table 1, produced more "diapause" moths as compared with the "non-diapause" hosts transplanted with the suboesophageal ganglion alone. From this it may be seen that the brain taken from the "diapause" donor weakly promotes the secretion of the suboesophageal ganglion through the nerve commissures. Transplanting the brain alone had no effect on voltinism, as in both FUKUDA'S and HASEGAWA'S experiments.

(c) *Inhibitory effect of brain taken from the "non-diapause" donor on the suboesophageal ganglion hormone in the "non-diapause" host.* The "non-diapause" hosts receiving the suboesophageal ganglion alone taken from the "non-diapause" donor (as seen in nos. 4 and 5 of Table 1) produced more "diapause" moths as compared with the "non-diapause" hosts receiving the brain-suboesophageal ganglion complex. In the former case some batches consisting wholly of diapause eggs were observed, as shown in Fig. 6, whereas in the latter case mixed egg batches only were observed, as shown in Fig. 7. This may be due to the fact that the brain taken from the "non-diapause" donor weakly inhibits the secretion of the suboesophageal ganglion through the nerve commissures in the "non-diapause" host.

(d) *Race differences in the suboesophageal ganglion and promoting function of the brain of the univoltine race.* It is seen from both no. 2 (bivoltine Diazō race) and no. 6 (univoltine European no. 16 race) of Table 1 that the function of the suboesophageal ganglion differs with each voltine race (see Figs. 9 and 10). Results of transplanting the suboesophageal ganglion alone showed less diapause egg batches as compared with that of the brain-suboesophageal ganglion complex, as shown in nos. 6 and 7 of Table 1. In the former case many mixed egg batches were observed, as shown in Fig. 10. But in the latter case many batches consisted wholly of

TABLE 2—MODIFICATION OF AMOUNT OF SILK AND COCOON WEIGHT BY TRANSPLANTATION OF THE SUBOESOPHAGEAL GANGLION IN DAIZŌ RACE

No.	Recipient	Donor	Transplanted					
			on 1/V		on 3/V		on 5/V	
	Daizō race	Daizō race	Cocoon weight (cg)	Silk (cg)	Cocoon weight (cg)	Silk (cg)	Cocoon weight (cg)	Silk (cg)
1.	Control	(Wounded)	88·7	7·6	86·7	7·4	85·6	7·4
2.	L ←—	H (SG)	95·4	8·4	94·3	9·0	86·9	7·5
3.	L ←—	H (Br + SG)	97·2	9·0	89·7	8·1	85·8	7·8
4.	L ←—	L (SG)	98·0	9·0	91·0	8·1	85·7	8·1
5.	L ←—	L (Br + SG)	95·7	8·4	93·1	7·9	86·5	7·5

Note: for abbreviations, see Table 1.

diapause eggs, as shown in Fig. 11. This fact is due to the presence or absence of a brain of the univoltine race.

(e) *Increase of amount of silk and of body weight by transplanting the suboesophageal ganglion and the brain-suboesophageal ganglion complex.* The function of the suboesophageal ganglion is related not only to the production of diapause egg batches, but also to the increase of silk and body weight (Table 2). The same facts were observed by NAGATOMO (1956) in transplantation experiments with the brain-suboesophageal ganglion complex.

(II) *Production of non-diapause eggs by transplanting the corpora allata and the brain-corpora allata complex*

(a) *Influence of larval age on the function of the transplanted corpora allata.* When the corpora allata and the brain-corpora allata complex taken from the "diapause" or "non-diapause" donors of Daizō race are transplanted into the "diapause" host of the same race, some "diapause" hosts lay either non-diapause egg batches or egg batches mixed with diapause and non-diapause eggs, as seen in nos. 1–5 of Table 3. The function of the corpora allata on the production of non-diapause eggs was well observed in the weak diapause hosts in which egg

VOL. 3 1959

TABLE 3—PRODUCTION OF NON-DIAPAUSE EGGS BY TRANSPLANTATION OF BOTH THE CORPORA ALLATA (CA) AND THE BRAIN-CORPORA ALLATA COMPLEX (Br+CA)

No.	Recipient (Daizō race)	Donor	(Race)	Transplanted on 1/V ~ 2/V: Non-diapause moths	Moths laid mixed eggs	Diapause moths	Transplanted on 3/V: Non-diapause moths	Moths laid mixed eggs	Diapause moths	Transplanted on 4/V ~ 5/V: Non-diapause moths	Moths laid mixed eggs	Diapause moths
1.	Control	(Wounded)		0	10	25	0	11	31	0	8	44
2.	H ←—	H (CA, 2)		4	13	21	6	9	23	0	3	25
3.	H ←—	H (Br+CA)	Di	3	8	19	4	7	24	0	4	23
4.	H ←—	L (CA, 2)		6	19	16	7	8	25	0	3	18
5.	H ←—	L (Br+CA)		4	19	8	16	10	9	1	7	25
6.	Control	(Wounded)		–	—	—	0	21	23	–	–	—
7.	H ←—	H (CA, 2)	3m	–	—	—	24	11	5	–	–	—
8.	H ←—	H (Br+CA)		–	—	—	14	18	9	–	–	—
9.	H ←—	H (CA, 2)		–	—	—	14	13	21	–	–	—
10.	H ←—	H (Br+CA)	mv	–	—	—	32	16	11	–	–	—

Note: L, low incubation at 15°C; H, high incubation at 24°C; 1/V, 2/V, 3/V, 4/V, 5/V, transplantation on the first, second, third, fourth, and fifth day of the fifth instar, respectively; Di, Daizō race; 3m, trimoulting; mv, multivoltine; (CA, 2), two corpora allata.

batches of many diapause eggs mixed with a few non-diapause eggs are produced. The function, however, could not be found in strong diapause hosts even if in the Daizō race.

The non-diapause eggs were produced more numerously by transplanting the corpora allata and the brain-corpora allata complex in the earlier stage than in the later of the fifth larval instar, as seen in nos. 1–5 of Table 3 (see Figs. 12–14). The transplantation of the corpora allata after the fourth day of the fifth instar showed no effect on the production of non-diapause eggs.

(b) *Inhibitory function of the brain taken from the "diapause" donor on the corpus allatum hormone in the "diapause" host.* The "diapause" hosts transplanted with the corpora allata alone taken from the "diapause" donor, as seen in nos. 2 and 3 of Table 3, produced more "non-diapause" moths as compared with the case of the "diapause" hosts transplanted with the brain-corpora allata complex. This is the reason why the brain extracted from the "diapause" donor weakly inhibits the secretion of the corpora allata through the nerve commissures in the "diapause" host.

(c) *Promoting function of the brain taken from a "non-diapause" donor on the corpus allatum hormone in the "diapause" host.* The "diapause" hosts transplanted with the corpora allata alone extracted from the "non-diapause" donor, as seen in nos. 4 and 5 of Table 3 (see Figs. 13 and 15), produced less "non-diapause" moths as compared with the case of the "diapause" hosts transplanted with the brain-corpora allata complex. This is due to the fact that the brain extracted from the "non-diapause" donor weakly promotes the secretion of the corpora allata through the nerve commissures in the "diapause" host.

(d) *Race differences in the function of the corpus allatum.* The function of the corpora allata in the tetramoulting race (no. 9) is weaker than that of the corpora allata in the trimoulting (no. 7), as shown in Table 3 (see Figs. 17 and 19).

(e) *Antagonistic function of the brain extracted from univoltine and multivoltine races on the corpus allatum hormone.* The "diapause" hosts of Daizō race (no. 7) transplanted with the corpora allata alone extracted from univoltine (trimoulting) donors produced more "non-diapause" moths than in the case of the hosts (no. 8) transplanted with the brain-corpora allata complex. Thus, the brain of the univoltine race weakly inhibits the secretion of the corpora allata through the nerve commissures (see Figs. 17 and 18) in the brain. On the other hand, the brain of the multivoltine race weakly promoted the secretion of the corpora allata (see Figs. 19 and 20). This follows from the fact that the "diapause" hosts (no. 9) transplanted with the corpora allata alone taken from multivoltine donors produced less "non-diapause" moths than in the case of the hosts (no. 10) transplanted with the brain-corpora allata complex (Table 3).

(f) *Decrease of silk and body weight by transplanting the corpora allata and the brain-corpora allata complex.* The "diapause" hosts (nos. 2 and 3 of Table 4) transplanted with the corpora allata or the brain-corpora allata complex produced less silk and their body weight was less as compared with those of the controlled series (no. 1).

TABLE 4—MODIFICATION OF AMOUNT OF SILK AND COCOON WEIGHT BY TRANSPLANTATION OF THE CORPORA ALLATA IN DAIZŌ RACE

No.	Recipient	Donor	Transplanted					
			on 1/V		on 3/V		on 5/V	
	Daizō race	Daizō race	Cocoon weight (cg)	Silk (cg)	Cocoon weight (cg)	Silk (cg)	Cocoon weight (cg)	Silk (cg)
1.	Control	(Wounded)	126·5	14·5	125·4	13·9	124·0	13·1
2.	H ←—	L (CA,2)	121·5	12·3	122·0	13·0	120·6	12·1
3.	H ←—	L (Br+CA)	121·0	12·3	121·0	12·4	118·9	12·3

(III) *Transplanting the suboesophageal ganglion with the corpus allatum*

Both the corpus allatum and the suboesophageal ganglion extracted from the donors on the first (1/V) and the fifth day (5/V) of the fifth larval instar were reversely transplanted into the young (1/V) and the old (5/V) hosts, as shown in Table 5.

TABLE 5—EFFECTS OF THE SUBOESOPHAGEAL GANGLION AND THE CORPORA ALLATA WHICH ARE INFLUENCED BY THE AGE OF RECIPIENTS AND DONORS

Group	Recipient	Donor	Cocoon weight (cg)	Silk (cg)	Number of non-diapause moths	Number of moths laid mixed eggs	Number of diapause moths
	Daizō race	Daizō race					
A	Control	(Wounded)	87·7	8·9	53	0	0
	L (1/V) ←—	H (SG, 1/V)	89·2	9·4	27	3	0
	L (1/V) ←—	H (SG, 5/V)	88·9	9·1	15	11	5
B	Control	(Wounded)	87·5	8·7	46	0	0
	L (5/V) ←—	H (SG, 1/V)	88·8	9·0	9	15	5
	L (5/V) ←—	H (SG, 5/V)	88·0	8·9	11	19	7
C	Control	(Wounded)	106·9	12·8	0	9	42
	H (1/V) ←—	L (CA, 1/V)	103·3	12·1	6	18	7
	H (1/V) ←—	L (CA, 5/V)	104·8	12·3	0	13	9
D	Control	(Wounded)	109·4	13·7	0	6	41
	H (5/V) ←—	L (CA, 1/V)	104·4	12·8	1	10	19
	H (5/V) ←—	L (CA, 5/V)	106·5	13·2	0	4	26

Note: for abbreviations, see Table 3.

From the results of Table 5 the following conclusions are drawn:

(1) The suboesophageal ganglion increases cocoon weight and amount of silk, and favours diapause, as seen from A and B groups.

(2) The transplantation of the suboesophageal ganglion is more effective for the increase of cocoon weight and amount of silk in the younger stage of the fifth instar than in the older, as observed in A and B groups.

(3) In the transplantation experiments at the earlier stage of the fifth instar, the old suboesophageal ganglion is more effective for the production of diapause eggs than the young one. In the later stage, however, the above-mentioned fact is not clear.

(4) The corpora allata decrease cocoon weight and amount of silk and favour non-diapause, as seen in C and D groups.

(5) In the transplantation experiments at the earlier stage of the fifth instar, the young corpora allata are more effective for the production of non-diapause eggs than the old ones. In the later stage, however, the young corpora allata have little effect, but the old corpora allata have no effect.

(IV) *Removal of the brain from "diapause" and "non-diapause" pupae*

When "diapause" pupae had their brains removed, some of the resulting moths laid non-diapause egg batches; when "non-diapause" pupae had their brains removed, some of them laid diapause egg batches. These facts agree with those obtained by FUKUDA (1952) and HASEGAWA (1952).

(V) *Function of the suboesophageal ganglion and the brain-corpora allata complex in multivoltine Cambodge hosts*

The bivoltine Daizō race was used as host in the previous experiments, whereas the multivoltine Cambodge race was used in the following ones. Cambodge race was maintained at 17°C during the first term of incubation and at 24°C during the second term (from the fourth day before hatching to hatching). Cambodge hosts thus dealt with are to lay the mixed diapause and non-diapause batches of eggs. The suboesophageal ganglion and the brain-corpora allata complex extracted from males and females of the bivoltine race were transplanted into the ventral side of the abdomen of the larval hosts. Results obtained are shown in Table 6.

From Table 6 the capacity of the suboesophageal ganglion to produce diapause eggs seems to be stronger in females than in males, as also shown in NAGATOMO's

TABLE 6—TRANSPLANTATION OF THE SUBOESOPHAGEAL GANGLION AND THE BRAIN-CORPORA ALLATA COMPLEX EXTRACTED FROM THE BIVOLTINE RACE INTO THE MULTIVOLTINE CAMBODGE HOSTS

No.	Recipient	Donor	Diapause moths		Non-diapause moths		Moths laid mixed eggs		% of non-diapause moths	% of non-diapause moths except moths laid mixed eggs
	Cambodge race	Bivoltine race	Number	%	Number	%	Number	%		
1.	Control	(Wounded)	27	21·6	43	34·4	55	44·0	50·4	61·4
2.	L.H. ←—	H (SG, ♀)	32	41·0	21	26·9	25	32·1	39·7	39·6
3.	L.H. ←—	H (SG, ♂)	36	36·0	28	28·0	36	36·0	41·0	43·8
4.	L.H. ←—	H (Br+CA, ♀)	6	6·7	35	39·3	48	53·9	68·5	85·4
5.	L.H. ←—	H (Br+CA, ♂)	3	3·5	39	45·9	43	50·6	64·7	92·9

Note: L.H., the first term of incubation was maintained at low temperature, 17°C, and the second term (from the fourth day before hatching to hatching) at high temperature, 24°C; H, high incubation at 24°C.

experiments (1956). The capacity of the corpora allata to produce non-diapause eggs was the same as in the Daizō race.

DISCUSSION

The production of diapause eggs by transplanting the suboesophageal ganglion has been found independently by FUKUDA (1951, 1952) and by HASEGAWA (1951, 1952) in the silkworm: HASEGAWA concluded that the voltinism is determined by the amount of the suboesophageal ganglion hormone. FUKUDA, however, claimed that the voltinism is determined by the pupal brain, which controls the secretion of the suboesophageal ganglion. When the suboesophageal ganglion hormone is not secreted at all in consequence of the inhibitory function of the brain through the nerve commissures, moths lay non-diapause egg batches. When a large quantity of hormone is secreted by the promoting function of the brain, moths produce diapause egg batches.

In the experiments of the author and his collaborators, however, the "non-diapause" hosts transplanted with the brain-suboesophageal ganglion complex extracted from the "non-diapause" donor produced some "diapause" moths which laid batches of mixed diapause and non-diapause eggs, as seen in no. 5 of Table 1. Repeated experiments show that this is always the case. If the brain in the "non-diapause" pupa perfectly inhibits the secretion of the suboesophageal ganglion, then "non-diapause" moths only should be produced in the above experiments. Therefore the inhibitory function of the brain of "non-diapause" individual must needs be weak.

VOL. 3 1959

FUKUDA'S hypothesis explains very well the case which converts the non-diapause egg into the diapause one. But it may not explain the case which converts the diapause egg to the non-diapause one. The brain of the diapause larva accelerates the function of the suboesophageal ganglion which secretes the diapause hormone. When the brain of this larva is removed, the reason that the non-diapause eggs were produced cannot be explained as above, for the suboesophageal ganglion alone has the capacity to convert non-diapause eggs into diapause ones.

The voltinism in the silkworm is, however, too complex to be explained by the action of the suboesophageal ganglion hormone alone. MOROHOSHI (1956, 1957) succeeded in producing non-diapause eggs by transplanting the corpora allata into "diapause" hosts. From these transplantation experiments, it was verified that the voltinism is determined by a balance of the corpora allata and suboesophageal ganglion hormones, and that the brain is weakly related to the secretory activities of the two hormones. The brain of the univoltine race or the brain of the "diapause" larva weakly promotes the secretory activity of the suboesophageal ganglion and weakly inhibits that of the corpora allata. On the other hand, the brain of the multivoltine race or the brain of the "non-diapause" larva weakly inhibits the secretory activity of the suboesophageal ganglion and weakly promotes that of the corpora allata.

When the suboesophageal ganglion is transplanted into a larval body, the ratio of corpora allata to suboesophageal ganglion (CA/SG) becomes small and the hosts

diapause; but, on the contrary, when the corpora allata are transplanted, the ratio of CA/SG becomes large and the hosts do not diapause.

The removal of the brain from the "diapause" pupa makes the ratio of CA/SG large; but, on the contrary, the removal from the "non-diapause" pupa makes the ratio of CA/SG small. Some moths in the former case lay non-diapause egg batches and some in the latter diapause ones.

The reason that the voltinism of the bivoltine race is controlled by temperature and light during incubation may be due to the change in the function of the young brain. When the silkworm is incubated under low temperatures and in darkness, the function of the brain will be changed, diapause → non-diapause; but, on the contrary, under high temperatures and in light, non-diapause → diapause.

The above-mentioned relations are illustrated in Figs. 1–3.

SUMMARY

Production of diapause eggs by transplantation of the suboesophageal ganglion or the brain-suboesophageal ganglion complex

(1) It was found from the transplantation experiments in the silkworm that the suboesophageal ganglion produces a hormone which converts the "non-diapause" individual into the "diapause" one.

(2) From the transplantation experiments in the fifth larval instar, the function of the suboesophageal ganglion seems to become gradually stronger with age. At the earlier stage of the fifth instar, the production of the "diapause" moth is more effective when the suboesophageal ganglion extracted from the old donor is employed than in the case from the young one. At the later stage, however, there is little difference between young and old donors.

(3) The function of the suboesophageal ganglion differs with each voltine race, and it is stronger in the univoltine than in the bivoltine.

(4) The brain controls the secretion of the suboesophageal ganglion through the nerve commissures. That is, the brain extracted from the "diapause" or from the univoltine pupa weakly promotes the secretion of the suboesophageal ganglion, whereas the brain extracted from the "non-diapause" or from a multivoltine pupa weakly inhibits the secretion of the suboesophageal ganglion.

(5) In the hosts transplanted with the suboesophageal ganglion or the brain-suboesophageal ganglion complex, the cocoon weight and amount of silk are large as compared with those of the controls.

Production of non-diapause eggs by transplantation of the corpora allata or the brain-corpora allata complex

(6) It was found from the transplantation experiments that the corpora allata produce a hormone which converts the "diapause" individual into the "non-diapause" one.

(7) From the transplantation experiments during the fifth instar, the capacity of the corpora allata became gradually weaker with age. At the earlier and the

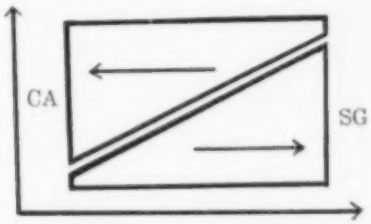


FIG. 1. Diagram illustrating the effect of age on the relative functions of the CA and SG organs in the fifth larval instar in the silkworm.

Ordinate, functions of hormones; abscissa, developmental stage. The function of the CA becomes weaker with age and that of the SG stronger. The two hormones act antagonistically to each other.

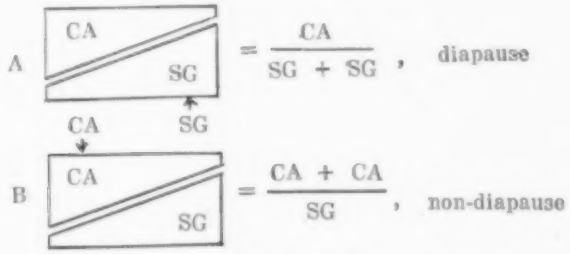


FIG. 2. Diagram illustrating the change of voltinism caused by transplantation of the CA and SG organs.

A, production of the diapause eggs by transplantation of the SG organ; B, production of the non-diapause eggs by transplantation of the CA organ.

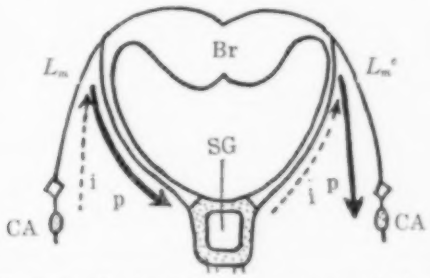


FIG. 3. Diagram illustrating the internal secretory organs which are found in the larval head of the silkworm, and their antagonistic relations.

SG, suboesophageal ganglion; CA, corpora allata; Br, brain; i, inhibition; p, promotion; L_m, brain of "diapause" pupa; L_m^e, brain of "non-diapause" pupa. The function of the CA is strongest in the trimoulting and weakest in the pentamoulting races; and the function of the SG is strongest in the univoltine and weakest in the multivoltine races. The brain (L_m) of "diapause" pupae weakly promotes (p) the function of the SG and weakly inhibits (i) the function of the CA. On the other hand, the brain (L_m^e) of "non-diapause" pupae weakly promotes the function of the CA and weakly inhibits the function of the SG. A growth-promoting hormone which is secreted from the CA acts antagonistically on a growth-inhibitory hormone which is secreted from the SG. Accordingly, the voltinism in the silkworm is determined by an antagonistic balance of these two hormones.

VOL. 3 1959

later stages of the fifth instar, the production of the "non-diapause" moth is more effective when corpora allata are taken from young rather than old donors.

(8) The capacity of the corpora allata differs in each moulting race: it is stronger in trimoulting than in tetramoulting races.

(9) The brain controls the secretion of the corpora allata through the nerve commissures. Namely, the brain extracted from the "diapause" or from the univoltine donor weakly inhibits the secretion of the corpora allata; on the other hand, the brain extracted from the "non-diapause" or from the multivoltine weakly promotes the secretion of the corpora allata.

(10) In the host transplanted with the corpora allata or the brain-corpora allata complex, the cocoon weight and amount of silk are less as compared with those of the controls.

Function of the suboesophageal ganglion and the corpus allatum

(11) The function of the suboesophageal ganglion and the corpora allata are related not only to the voltinism, but also to quantitative characters such as larval duration, moult-numbers, cocoon weight, and amount of silk. The suboesophageal ganglion tends to increase these characters, whereas the corpora allata tend to decrease them. It seems reasonable to believe that the suboesophageal ganglion hormone may be a growth-inhibitory hormone, and the corpus allatum a growth-promoting one.

REFERENCES

FUKUDA S. (1951) The production of the diapause eggs by transplanting the suboesophageal ganglion in the silkworm. *Proc. imp. Acad. Japan* **27**, 672–677.

FUKUDA S. (1952) Function of the pupal brain and suboesophageal ganglion in the production of non-diapause and diapause eggs in the silkworm. *Annot. zool. jap.* **25**, 149–155.

HASEGAWA K. (1951) Studies on the voltinism in the silkworm, *Bombyx mori* L., with special reference to the organs concerning determination of voltinism. A preliminary note. *Proc. imp. Acad. Japan* **27**, 667–671.

HASEGAWA K. (1952) Studies on the voltinism in the silkworm, *Bombyx mori* L., with special reference to the organs concerning determination of voltinism. *J. Fac. Agri. Tottri Univ.* **1**, 83–124.

MOROHOSHI S. (1957) Physiogenetical studies on moltinism and voltinism in *Bombyx mori*; a new hormonal antagonistic balance theory on the growth. *Jap. Soc. Prom. Sci., Tokyo* 1–202.

MOROHOSHI S., KIKUCHI R., and MIYAZAWA H. (1957) Studies on the voltinism in the silkworm, *Bombyx mori*. VI. Production of the diapause eggs by transplanting the suboesophageal ganglion and brain-suboesophageal ganglion complex. *J. seric. Sci. Tokyo* **26**, 232–233. (In Japanese).

MOROHOSHI S., MIYAZAWA H., and KIKUCHI R. (1956) Production of the non-diapause eggs by transplanting the corpora allata and brain-corpora allata complex in the silkworm, *Bombyx mori*. *Jap. J. Genet.* **31**, 305. (In Japanese.)

MOROHUSHI S., MIYAZAWA H., and KIKUCHI R. (1957) Studies on the voltinism in the silkworm, *Bombyx mori*. VII. Functions of the corpora allata and suboesophageal ganglion. *J. Seric. Sci. Tokyo* **26**, 233. (In Japanese.)

NAGATOMO T. (1956) Functions of the brain-suboesophageal ganglion complex in the silkworm, *Bombyx mori*. *Proc. imp. Acad. Japan* **32**, 500–503.

APPENDIX

EXPLANATION OF PLATE

ALL the figures are photographs of the egg batches laid by silkworm moths. They were made under the same magnification. Table 7 shows the results obtained in the transplantation experiments designed in four possible combinations. In the photographs diapause eggs remain black and non-diapause hatched eggs (empty) remain white.

TABLE 7—CHANGES OF THE VOLTINISM BY TRANSPLANTATION OF THE SUBOESOPHAGEAL GANGLION (SG), THE BRAIN-SUBOESOPHAGEAL GANGLION COMPLEX (Br+SG), THE CORPORA ALLATA (CA) AND THE BRAIN-CORPORA ALLATA COMPLEX (Br+CA)

Group	No. of figures	Recipient (Daizō race)	Donor	
				(Race)
A	4.	Control	(Wounded)	Bivoltine Daizō
	5.	L ←—	L (SG, 1/V)	Bivoltine Daizō
	6.	L ←—	L (SG, 5/V)	Bivoltine Daizō
	7.	L ←—	L (Br+SG, 5/V)	Bivoltine Daizō
B	8.	Control	(Wounded)	Bivoltine Daizō
	9.	L ←—	H (SG, 1/V)	Bivoltine Daizō
	10.	L ←—	H (SG, 3/V)	Univoltine European No. 16
	11.	L ←—	H (Br+SG, 3/V)	Univoltine European No. 16
C	12.	Control	(Wounded)	Bivoltine Daizō
	13.	H ←—	L (CA, 2/V)	Bivoltine Daizō
	14.	H ←—	L (CA, 4/V)	Bivoltine Daizō
	15.	H ←—	L (Br+CA, 2/V)	Bivoltine Daizō
D	16.	Control	(Wounded)	Bivoltine Daizō
	17.	H ←—	H (CA, 2/V)	Univoltine trimoulting
	18.	H ←—	H (Br+CA, 2/V)	Univoltine trimoulting
	19.	H ←—	H (CA, 2/V)	Multivoltine tetramoulting Cambodge
	20.	H ←—	H (Br+CA, 2/V)	Multivoltine tetramoulting Cambodge

Note: L, low incubation at 15°C; H, high incubation at 24°C; 1/V, 2/V, 3/V, 4/V, 5/V, transplanted on the first, second, third, fourth, and fifth day of the fifth instar, respectively.

4–7 (group A). Influence of age of the suboesophageal ganglion (SG) on the function to produce the diapause eggs, and inhibitory function of the brain (Br) on the SG through the nerve commissures.

It is seen from Figs. 5 and 6 that the function of the SG becomes gradually stronger with age for the production of diapause eggs, and from Figs. 6 and 7 that the brain extracted from "non-diapause" donors weakly inhibits the secretion of the SG through the nerve commissures.

8–11 (group B). Race difference of the function of the SG, and promoting function of the brain on the SG through the nerve commissures.

It is seen from Figs. 9 and 10 that the function of the SG differs with each voltine race, and from Figs. 10 and 11 that the brain of univoltine European No. 16 weakly stimulates the secretion of the SG through the nerve commissures.

12–15 (group C). Influence of age of the corpora allata (CA) on the function to produce the non-diapause eggs, and promoting function of the brain on the CA through the nerve commissures.

It is seen from Figs. 13 and 14 that the function of the CA becomes gradually weaker with age for the production of non-diapause eggs, and from Figs. 13 and 15 that the brain extracted from "non-diapause" donors weakly stimulates the secretion of the CA through the nerve commissures.

16–20 (group D). Race difference of the function of the CA, and inhibitory or promoting function of the brain on the CA through the nerve commissures.

It is seen from Figs. 17 and 19 that the function of the CA differs with moulting race, and from Figs. 17 and 18 that the brain of "diapause" trimoulting race weakly inhibits the secretion of the CA through the nerve commissures. It is also clear from Figs. 19 and 20 that the brain extracted from multivoltine donors weakly stimulates the secretion of the CA through the nerve commissures. For further explanations see text.

VOL.
3
1959

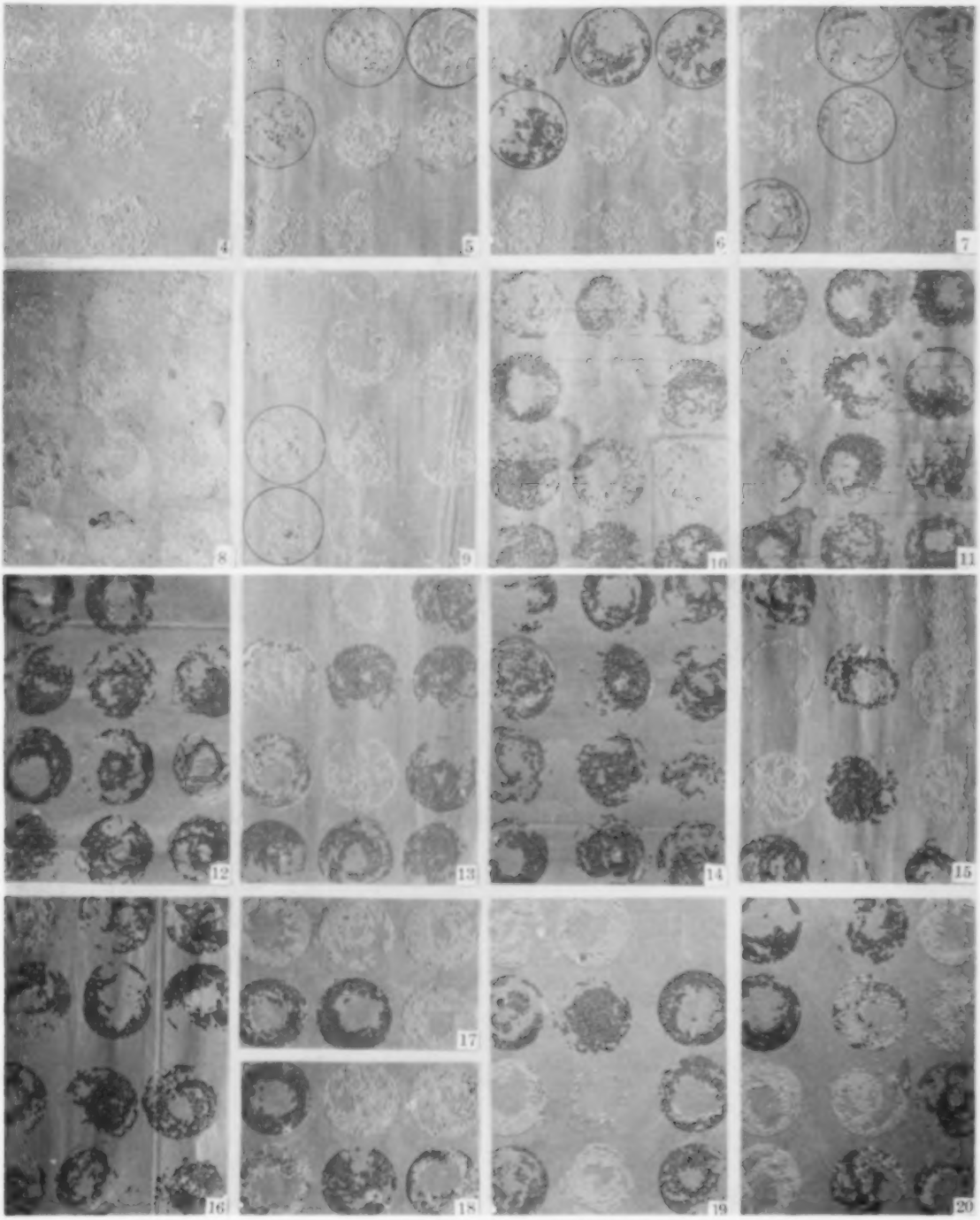


PLATE 1. FIGS. 4–20.

J. Ins. Physiol., 1959, *Vol. 3, pp. 41 to 49. Pergamon Press Ltd., London. Printed in Great Britain*

ELECTRICAL RESPONSE FROM THE COMPOUND EYE OF *LUCILIA**†

K. NAKA and M. KUWABARA

Department of Biology, Faculty of Science, Kyushu University

(*Received* 17 *May* 1958)

Abstract—ERGs were recorded from the compound eye of *Lucilia* with the aid of an ultra-microelectrode. As with *Apis*, the ERG from *Lucilia* was shown to consist of two components, positive and negative, which had their origin in the receptor layer. Two components were recorded from the region corresponding to the medulla externa in inverse polarity. The negative component reversed its polarity at the basement membrane, and it was concluded that this polarity reversal was an active process.

VOL.
3
1959

INTRODUCTION

THE electrical response from the compound eye of insects has been reported by many observers. The component analysis of the ERG from the insect compound eye was suggested by JAHN and WULFF (1942), AUTRUM and GALLWITZ (1951), and HASSENSTEIN (1957). According to JAHN and WULFF, the ERG from the compound eye of a grasshopper was the algebraic sum of two components, one from the retinal layer, and the other, of an opposite sign, from the optic ganglion. AUTRUM and GALLWITZ also reached nearly the same conclusion. On the other hand, KUWABARA and NAKA (1957) have recorded the ERG from several insects including *Apis* with the aid of a micro-electrode and concluded that the ERG consisted of two monophasic components with an opposite sign that had their origin in the receptor layer.

BURTT and CATTON (1956) have reported that the ERG from several insects reversed its polarity somewhere in the optic pathway, and they concluded that in *Calliphora* the reversal had occurred in the first synaptic region.

This paper will describe some features of the ERG from the compound eye of *Lucilia* and also deals with the polarity reversal of the ERG at the optic pathway.

MATERIALS AND METHODS

The fly, *Lucilia caesar*, was used as the experimental animal. Before the experiments, the head was detached from the body.

When the ERG from the retinal layer and the optic ganglion was studied the head was horizontally cut in half to expose the optic lobes. The preparation was fixed in a Ringer pool with Vaseline.

* Contribution from the Biological Institute, Faculty of Science, Kyushu University, No. 69.

† Aided in part by the Scientific Research Fund of the Ministry of Education.

To study the ERG from the excised receptor layer the retinal layer was cut off with a sharp razor. Thus the prepared material consisted of the retinal layer and the basement membrane. Occasionally the remnants of the optic ganglion were involved in the preparation but this did not cause any change in the results obtained by this method of recording the ERG. The methods of recording the ERG from the excised retinal layer will be described later.

The light source was a 250 w projector lamp, and a lens was employed to focus light from the lamp on the preparation. The stimulating light was flashed on the preparation by a focal plane shutter with a fixed exposure time of about 50 msec.

The intensity of the stimulating light was controlled by three neutral filters, each of which had an adsorption of 90 per cent. The intensity of the stimulating light without filters will be referred to as 1 or unit intensity.

The electrode used was a 3μ KCl-filled capillary electrode with a tip diameter of less than 1M. The electrode was positioned and inserted into the preparation by a micro-manipulator.

As an input stage of the amplifier an electrometer tube was employed, as specified by ELMORE and SANDS (1949). Two D.C. amplifiers were used, one for the amplification of the action potential and the other for the amplification of the light signal. The amplified potential was displaced on the screen of a CRT and recorded with a 35 mm camera. From time to time a 0·5 μF condenser with a grid leak of 1 MΩ was inserted between the input stage and the following D.C. amplifier to prevent the drifting of the base line. No noticeable distortion of the wave form resulted from this C.R. coupling.

All the experiments were conducted in a room temperature ranging from 10°–15°C.

In the present paper 'ERG' will refer to electrical potential caused by illumination and recorded from points anywhere in the receptor layer or on the optic ganglion.

RESULTS

The head of the insect was horizontally cut in half to expose the retinal layer and the optic lobes. Under a binocular microscope an electrode was inserted into three regions, the retinal layer, the region proximal to the basement membrane, and the region corresponding to the medulla externa. The wave form of the ERG varied according to the region where the electrode was inserted.

ERG from fresh preparations

The ERGs recorded from the retinal layer of a fresh preparation are shown in Fig. 1. The response to illumination of a very low intensity (0·1 per cent of the unit intensity) was a monophasic positive wave (Fig. 1a). When the illumination was increased in intensity the wave form of the ERG became complicated (Fig. 1b and 1c). This seemed to be caused by the appearance of a negative component

VOL. 3 1959

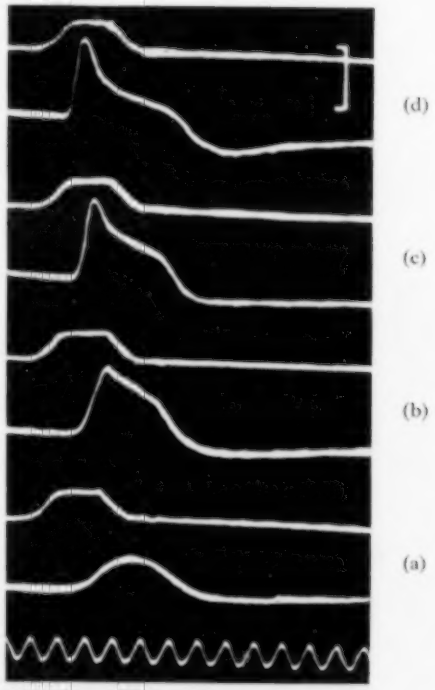


FIG. 1. ERGs from the retinal layer of a fresh preparation. Throughout this paper ERGs were recorded at different intensities of stimuli, 0·001, 0·01, 0·1 and 1·0 of unit intensity. The upper line of the record represents the stimulus signal. ERGs in Figs. 1–6 were recorded from the same preparation. The photographs are retouched.

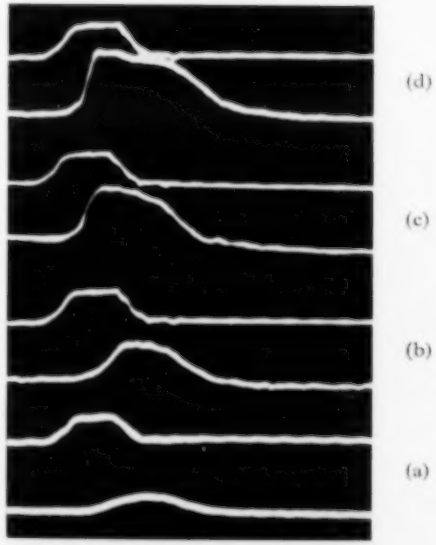


FIG. 2. ERGs from the region proximal to the basement membrane. Other legends are same as in Fig. 1.

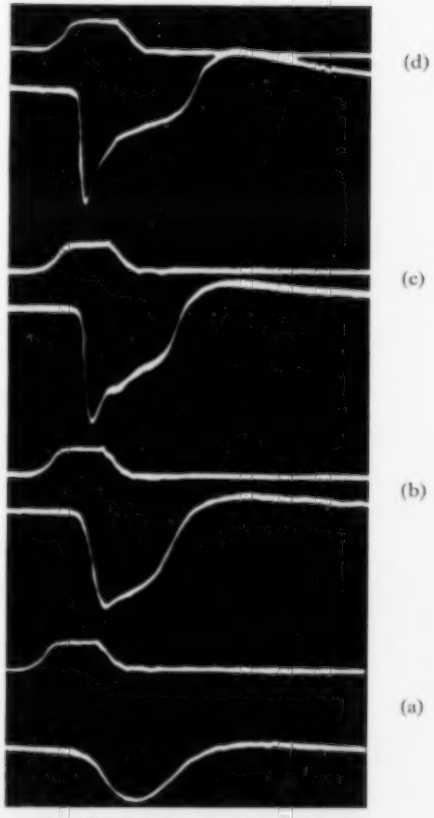


FIG. 3. ERGs from the medulla externa. Other legends are same as in Fig. 1.

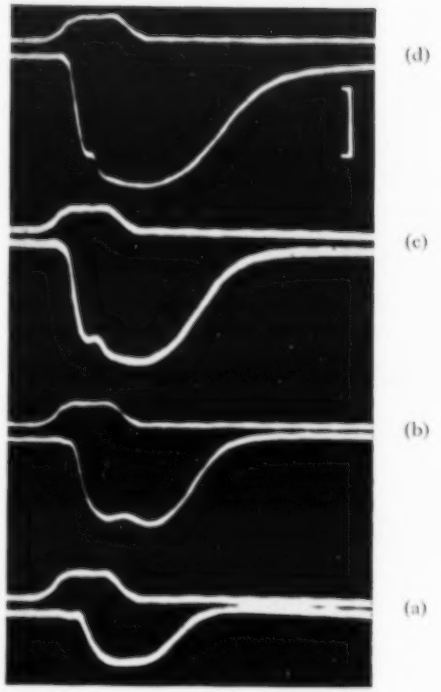


FIG. 4. ERGs from the receptor layer of a degenerating preparation. Reference voltage, 5 mV. Other legends are same as in Fig. 1.

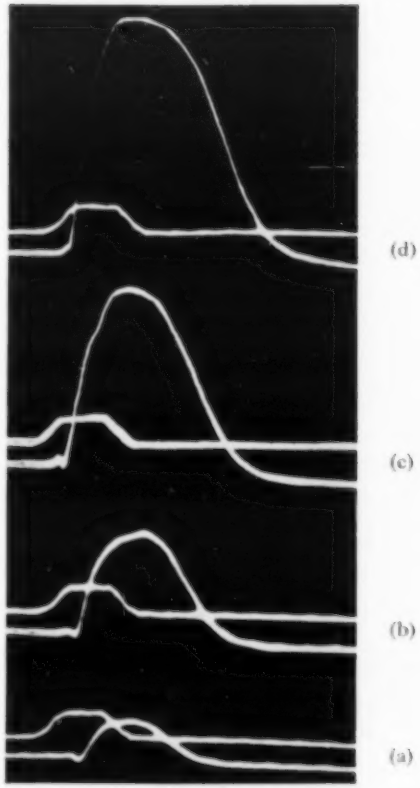


FIG. 5. ERGs from the region proximal to the basement membrane. Other legends are same as in Fig. 1.

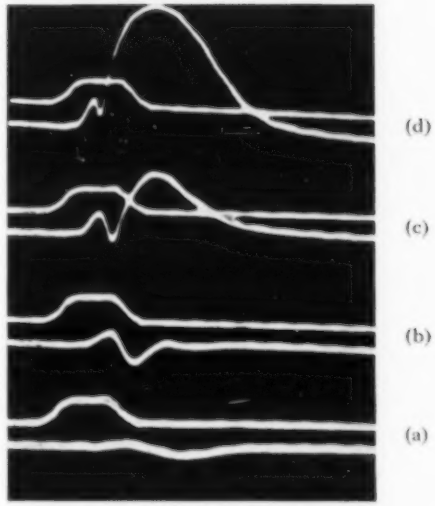


FIG. 6. ERGs from the medulla externa. Other legends are same as in Fig. 1.

in the ERG. The ERG which is shown in Fig. 1d has been reported by KUWABARA and NAKA (1957) to consist of two monophasic components with opposite polarity. The positive spike-like potential at the onset of the ERG evoked by a high intensity illumination seemed to be due to interaction between the two components. It is apparent that in the fresh preparation of the *Lucilia* compound eye the response to low intensity light stimulus consisted of a positive component while a high intensity light stimulus caused the appearance of a negative component in the ERG.

The ERGs recorded from the region just proximal to the basement membrane were monophasic positive waves which did not undergo any changes in the wave form when the illumination was increased in intensity (Fig. 2). Therefore the ERG from this region of the optic pathway seemed to consist of a single component which was positive in polarity.

The ERGs recorded from the region corresponding to the medulla externa are shown in Fig. 3. The wave form of the ERGs produced in this region are mirror images of the ERGs from the receptor layer. The response to the stimulation of a very low intensity (0·1 per cent of the unit intensity) was a negative wave (Fig. 3a) and with increase in the intensity of the illumination a positive component appeared in the ERG (Fig. 3b and 3c) and a negative spike-like potential, which was the counterpart of the positive spike-like potential in the ERG from the receptor layer, became more conspicuous. It is apparent that in this region of the optic lobe two components in the retinal layer were recorded in inverse polarity, the positive component in the retinal layer recorded as a negative wave and the negative one as a positive wave. When the electrode was moved from the medulla externa toward the central side of the preparation, the wave form of the ERG did not change although the amplitude of the response declined gradually.

VOL. 3 1959

ERG from degenerating material

The day after the preceding experiments, the ERGs were recorded from the same preparation under the same conditions. At that time the preparation seemed to be decaying. The regions where the electrode was inserted were the same as in the preceding experiments.

The ERGs recorded from the retinal layer were negative, as shown in Fig. 4. The response to illumination of a very low intensity (0·1 per cent of the unit intensity) was a monophasic negative wave and the increase in the intensity of the illumination caused the appearance of a positive hump in the ERG (Fig. 4b, c, d). The hump appeared at the earlier part of the response when the stimulus was increased in intensity. This type of response was quite different from the response of the fresh preparation. As it is inconceivable that the positive component from the retinal layer of the fresh preparation would reverse its polarity as a result of the degeneration, it seemed reasonable to suppose that the negative wave from the retinal layer of the degenerating material corresponded to the negative component in the ERG from the fresh preparation. Consequently the positive hump was the remnant of the positive component of the ERG from the fresh preparation.

Thus the difference between the two types of ERG (i.e. those from the fresh and degenerating preparations) seemed to have been brought about by the degeneration which resulted in increase in the amplitude of the negative component and decrease in the amplitude of the positive component. At the same time it is interesting to note that in the degenerating material the negative component preceded the positive one whereas in the fresh preparation the positive one preceded the negative component.

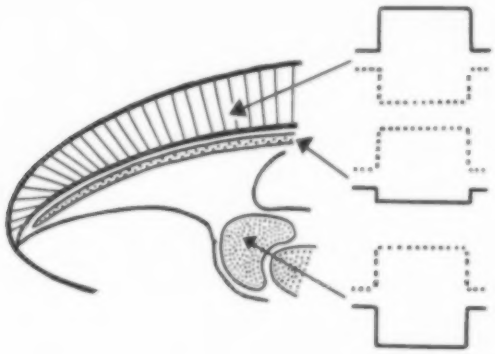


FIG. 7. Analysis of the ERG from the receptor layer and the optic pathway of *Lucilia*. ERGs recorded from the receptor layer, the region proximal to the basement membrane and the medulla externa are schematically separated into two components. The full line and dotted line represent the positive and negative component in the receptor layer respectively.

When the preparation further degenerated, the positive hump disappeared entirely from the ERG although the negative component regenerated in the same amplitude for three or four days under the experimental conditions. The ERGs from the region proximal to the basement membrane were positive waves as in the fresh preparation although the amplitude of the responses was greatly increased (Fig. 5). The hump was present but had a very small amplitude; the polarity of the hump was also reversed. Judging from the wave form of the ERG, the large positive wave and the negative hump corresponded respectively to the negative wave and the positive hump in the ERG from the receptor layer.

The ERGs recorded from the region corresponding to the medulla externa are shown in Fig. 6. The ERG recorded with a high intensity flash light, consisted of a positive wave and a negative hump which had considerable amplitude. The ERG in Fig. 6d was a mirror image of the ERG from the retinal layer. When the stimulation was decreased in intensity the amplitude of the positive wave became smaller in comparison with the negative hump.

From the above results the relation between the two components from the retinal layer can be summarized as follows: the ERG from the retinal layer of the insect compound eye is composed of two components, a positive and a negative

wave. The positive component appears in the medulla externa as a negative wave, although it can be recorded in very small amplitude from the region proximal to the basement membrane. The negative component from the retinal layer reverses its polarity at the basement membrane and is recorded from the optic pathway as a positive wave. These relations are shown schematically in Fig. 7.

The polarity reversal of the negative component at the basement membrane

In the preceding experiments it was shown that the negative component from the retinal layer reversed its polarity at the basement membrane. The same phenomenon has also been observed by NAKA and KUWABARA (1959) on *Procambarus*.

To investigate the polarity reversal of the negative component in detail the ERG from the excised receptor layer was recorded by an electrode which was inserted through the basement membrane toward the corneal side. The method of recording the ERG is shown schematically in Fig. 8.

VOL. 3 1959

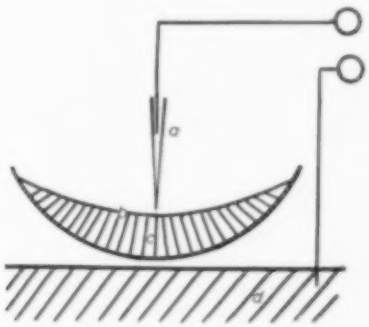


FIG. 8. Schematic representation of the method of recording the ERG from the dissected receptor layer. (a) microelectrode, (b) basement membrane, (c) receptor layer and (d) cotton wick.

The ERG recorded from the electrode just touching the surface of the basement membrane was a large monophasic positive wave which had the same wave form as the ERG from the proximal region of the basement membrane of the degenerating material (Fig. 9). The increase in the intensity of the illumination resulted only in the increase in the amplitude and duration of the response but not in any change in the wave form. The electrode was pushed downward through the basement membrane and the ERGs recorded from this position of the electrode are shown in Fig. 10. The response to the illumination of very low intensity (0·1 per cent of the unit intensity) was a negative wave which seemed identical with the negative wave from the receptor layer of the degenerating material. When the illumination was increased in intensity there appeared a positive wave in the ERG (Fig. 10c). The response to illumination of the unit intensity was a triphasic wave which consisted of a negative wave and a positive wave (Fig. 10d). The positive component which appeared in the high intensity response seemed to correspond

to the positive wave recorded by the electrode placed on the surface of the basement membrane. When the electrode was inserted more deeply toward the corneal surface the positive wave which appeared in the response to the illumination of high intensity became smaller and the negative wave dominated the ERG (Fig. 11). The further insertion of the electrode caused the disappearance of the positive wave and the ERG consisted of a negative component which was identical with the negative wave recorded from the receptor layer of the degenerating material (Fig. 12).

DISCUSSION

The ERG has been recorded from the compound eye of insects by many authors. JAHN and WULFF (1942) reported that the ERG from the grasshopper consisted of two components. They supposed that the ganglion was the site of an ERG component opposed to the negative potential from the receptor layer. AUTRUM and GALLWITZ (1951) also reached nearly the same conclusion. According to them, the illumination potential was divisible into two components, a negative generator potential from the retinal layer and a positive one from the optic lobe.

On the other hand, KUWABARA and NAKA (1957) have shown that the ERG from the compound eye of several insects, including *Apis*, consisted of two monophasic components, and they concluded that the two components had their origin in the receptor layer. As in *Apis*, the response of the fresh preparation of *Lucilia* to a stimulus of a very low intensity was a positive wave. Although the increase in the intensity of the illumination had caused the appearance of a negative component in the ERG, the positive component always preceded the negative component. Judging from the results of the present experiment it is unlikely that the positive component was generated by the negative potential. Moreover, the ERG was recorded with an ultra-microelectrode which suggests that the recorded ERG was from a very restricted area. On the other hand the negative component preceded the positive component in the degenerating material. The observation that the latency of two components could be interchangeable leads to the conclusion that the two components originated independently in the retinal layer. KUWABARA and NAKA (1957) reached the same conclusion on *Apis*.

The positive component was easily susceptible to degeneration and with the progress of the degeneration it became smaller until it disappeared entirely from the ERG. On the other hand, the negative component was so resistant to degeneration that it was still recorded three days after decapitation. It is interesting to note that the positive component of the ERG from the receptor layer of *Procambarus* was found to be more susceptible to degeneration than the negative component (NAKA and KUWABARA, 1956, 1959).

It seems inconsistent that in the fresh preparation the response to the illumination of low intensity was a positive wave whereas in the degenerating preparation it was a negative wave. Furthermore, the amplitude of the negative wave was greater in the degenerating material than in the fresh one. This inconsistency

VOL.
3
1959

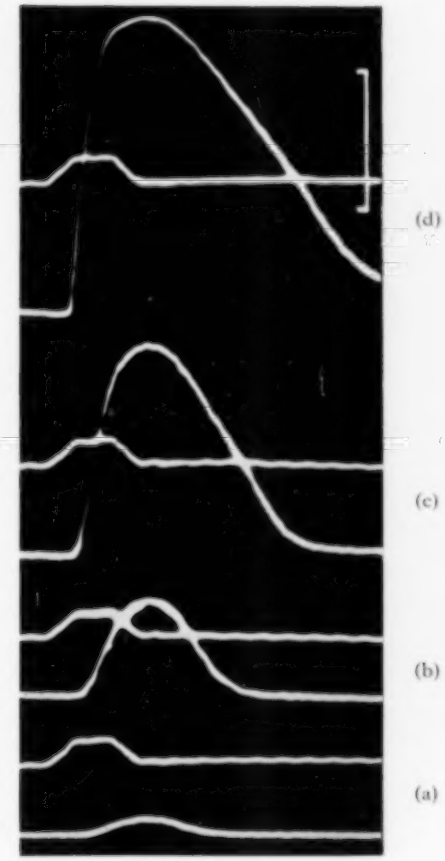


FIG. 9. ERGs recorded when the electrode touched the inner surface of the basement membrane of a dissected preparation. ERGs in Figs. 9–12 were recorded from the same preparation. Reference voltage, 5 mV. Other legends are same as in Fig. 1.

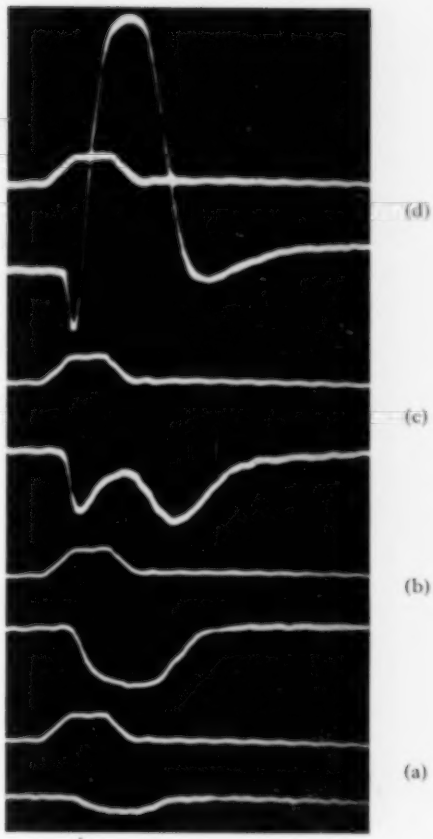


FIG. 10. ERGs recorded when the electrode was inserted into the basement membrane. Other legends are same as in Fig. 1.

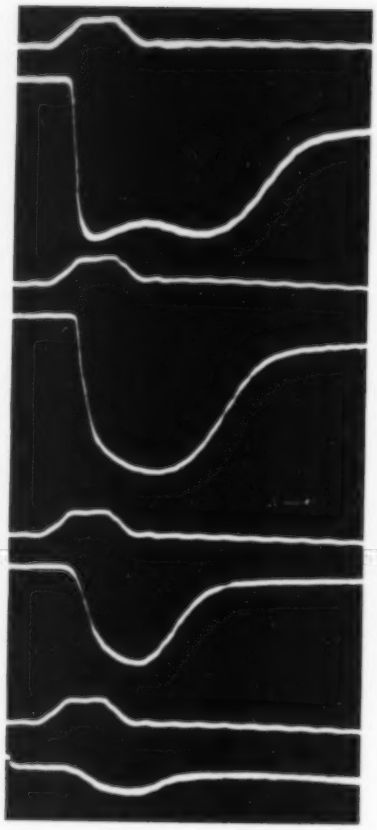


FIG. 11. ERGs recorded when the electrode was inserted through the basement membrane. Other legends are same as in Fig. 1.

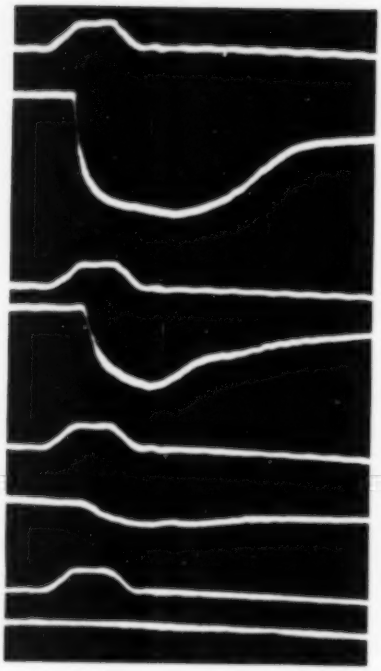


FIG. 12. ERGs recorded when the electrode was in the receptor layer. Other legends are same as in Fig. 1.

can be explained if it is postulated that the full generation of the negative component from the receptor is, under normal conditions, inhibited by some mechanism which is made ineffective by degeneration. HASSENSTEIN (1957) recorded the ERG from *Sarcophaga* and *Eristalis* and observed that the diphasic ERG was transformed into a monophasic wave after a period of degeneration. As to the component analysis of the ERG he suggested that the ERG was composed of two components, a diphasic wave and a negative one. AUTRUM (1950) and AUTRUM and GALLWITZ (1951) have classified the ERG from the compound eye of insects into two types, namely the slow and the fast. According to their classification the ERG from the fresh preparation of *Lucilia* belongs to the fast type, whereas degeneration changes it into the slow type. In other words the fast type consists of two components, the positive and negative, whereas the slow type consists of the negative component only.

The polarity reversal of the ERG in the optic lobe was observed by BURTT and CATTON (1956) in certain insects. In *Calliphora*, they concluded that the reversal had occurred at the first synaptic region. RUCK and JAHN (1954) have also reported that the ERG from *Ligia* reversed its polarity at the optic pathway and this reversal occurred even when the ganglion was absent. In *Lucilia*, the polarity reversal can be divided into two problems: the reversal of the ERG at the optic pathway, and the reversal of the negative component at the basement membrane. The inverted ERG was recorded from the region corresponding to the medulla externa, with the positive component in the retinal layer recorded as a negative wave, whereas the negative wave in the retinal layer was recorded as a positive one. This type of polarity reversal did not occur when the optic ganglion was removed. The fact that the amplitude of the inverted positive wave from the retinal layer was smaller at the region proximal to the basement membrane than at the medulla externa may offer a clue to explain this phenomenon.

In the degenerating preparation of *Lucilia* the negative wave recorded from the retinal layer reversed its polarity at the basement membrane. There are two explanations for the polarity reversal of the negative component at the basement membrane. The first is that the two waves, the negative wave from the receptor layer and the positive wave from the region proximal to the basement membrane, had the same origin of generation and the negative wave reversed its polarity at the basement membrane by a physical mechanism such as the high resistance of the basement membrane. This type of reversal may be called an inactive reversal. The other explanation is that the two components had different origins of generation, which may be called an active reversal. The results of the present experiments support the latter view inasmuch as when the electrode was inserted through the basement membrane there was a point at which the ERG was triphasic. The ganglion seems to play no part in the reversal of the negative component. The reversal of the ERG reported by RUCK and JAHN seems to belong to this type.

On the polarity reversal of the negative component at the basement membrane these results lead to the tentative hypothesis that the negative potential from the

receptor layer may induce a positive potential from the region proximal to the basement membrane at the region where the retinula cells penetrate the basement membrane.

In recent years the ultra-microscopic structure of the compound eye has been revealed by some writers (GOLDSMITH and PHILPOTT, 1957 ; WOLKEN, CAPENOS, and TURANO, 1957), but it is still difficult to correlate the results of the electrophysiological investigation with the possible structures in the retina. The present results suggest that some structure at the basement membrane may play an important role in the reversal of the negative component at the basement membrane. As to the origin of the ERG, BURTT and CATTON (1956) have presented the hypothesis that the origin of the illumination potential was confined to the first synaptic region. WULFF, FRY, and LINDE (1955) suggested that the retinal potential was generated by a substance called C which was produced when the light acted on a photosensitive substance S. HARTLINE and his collaborators (1952) have inserted the microelectrode into the *Limulus* ommatidia and recorded the intracellular retinal action potential, which was supposed to originate from the eccentric cell. On the other hand OTTOSON and SVAETICHIN (1952) also concluded that the ERG was generated only from the receptor layer in the frog's retina.

The present result supports the conclusion reached by KUWABARA and NAKA (1957) that the insect ERG is composed of two components which originate from the receptor layer.

Acknowledgements—The authors would like to thank Mr. H. MORITA for his help in the course of this work. The authors also wish to thank Miss K. SHIGETOMI for her assistance in the preparation of the manuscript.

REFERENCES

AUTRUM H. (1950) Die Belichtungspotentiale und das Sehen der Insekten. *Z. vergl. Physiol.* **32**, 176–227.

AUTRUM H. and GALLWITZ U. (1951) Zur Analyse der Belichtungspotentiale des Insektenauges. *Z. vergl. Physiol.* **33**, 407–425.

BURTT E. T. and CATTON W. T. (1956) Electrical response to visual stimulation in the optic lobes of the *Locust* and certain other insects. *J. Physiol.* **133**, 68–88.

ELMORE W. C. and SANDS M. (1949) *Electronics*. McGraw-Hill.

GOLDSMITH T. H. and PHILPOTT D. E. (1957) The microstructure of the compound eyes of insects. *J. biophys. biochem. Cytol.* **3**, 429–440.

HARTLINE H. K., WAGNER H. G., and MACNICHOL E. F. (1952) The peripheral origin of nervous activity in the visual system. *Cold Spring Harb. Symp. Quant. Biol.* **17**, 125–141.

HASSENSTEIN B. (1957) Uber Belichtungspotentiale in den Augen der Fliegen *Sarcophaga* und *Eristalis*. *J. ins. Physiol.* **1**, 124–130.

JAHN T. L. and WULFF V. J. (1942) Allocation of electrical response from the compound eye of grasshoppers. *J. gen. Physiol.* **26**, 75–88.

KUWABARA M. and NAKA K. (1957) ERG from the compound eye of the honeybee. *Zool. Mag., Tokyo* **66**, 112. (In Japanese.)

NAKA K. and KUWABARA M. (1956) The component analysis of the ERG from the compound eye of *Cambarus*. *Mem. Fac. Sci. Kyush Univ.* (*E*) **2**, 75–86.

NAKA K. and KUWABARA M. (1959) Two components of the ERG from the compound eye of the crayfish. *J. Exp. Biol.* In press.

OTTOSON D. and SVAETICHIN G. (1952) Electrophysiological investigations of the frog retina. *Cold Spring Harb. Symp. Quant. Biol.* **17**, 165–173.

RUCK P. and JAHN T. L. (1954) Electrical studies on the compound eye of *Ligia occidentalis Dana* (Crustacea: Isopoda). *J. gen. Physiol.* **37**, 825–849.

WOLKEN J. J., CAPENOS J., and TURANO A. (1957) Photoreceptor structures III. *Drosophila melanogaster*. *J. biophys. biochem. Cytol.* **3**, 441–448.

WULFF V. J., FRY W. J., and LINDE F. A. (1955) Retinal action potential-theory and experimental results for grasshopper eyes. *J. cell. comp. Physiol.* **45**, 247–263.

VOL.
3
1959

J. Ins. Physiol., 1959, *Vol. 3, pp. 50 to 56. Pergamon Press Ltd., London. Printed in Great Britain*

QUANTITATIVE UNTERSUCHUNG DER FREIEN AMINOSÄUREN UND POLYPEPTIDE WÄHREND DER EMBRYONALENTWICKLUNG VON *DROSOPHILA MELANOGASTER*

URSULA VON DER CRONE-GLOOR

Aus dem Zoologisch-vergleichend anatomischen Institut der Universität Zürich*

(*Received* 4 *June* 1958)

Abstract—The free amino acids and polypeptides were determined qualitatively and quantitatively with the aid of paper chromatography during the embryonic development of *Drosophila melanogaster*. The total ninhydrin positive material reaches its minimum shortly before hatching and subsequently increases. Aspartic acid is present in large amounts in freshly laid eggs but decreases during the first 24 hours until it nearly disappears. Glutamic acid increases at first but then later also decreases. The concentration of glutamine is inversely proportional to that of glutamic acid. α-alanine increases clearly and regularly during the entire embryonic development.

EINLEITUNG

FÜR die meisten embryonal wirkenden Letalfaktoren von *Drosophila melanogaster* kennen wir bis heute nur das morphologisch-histologische Schädigungsmuster. Um zu einem möglichst vollständigen Bild des pleiotropen Wirkungsmusters zu gelangen, sollten aber auch physiologische und biochemische Phäne erfasst werden (HADORN, 1955). BOELL und POULSON (1939) und BULL (1954) untersuchten vergleichend die Atmungsrate normaler und embryonal letaler Keime und stellten übereinstimmend fest, dass die Atmung bei den letalen Embryonen stark herabgesetzt ist. NAKAMURA *et al.* (1951a) chromatographierten Hydrolysate aus normalen, letalen (yy) und unbefruchteten Eiern und fanden einen nicht näher bestimmten Stoff (ev. Cystin) in den letalen und unbefruchteten Eiern, nicht aber in den normalen. Ähnliche Verhältnisse zeigten sich auch für *Drosophila virilis* NAKAMURA *et al.* (1951b). Dieselben Autoren stellten für die letalen Embryonen eines attached-X Stammes von *Drosophila melanogaster* einen Abfall gewisser Aminosäuren (z. B. Glutaminsäure, Asparaginsäure) gegenüber normalen Eiern fest (NAKAMURA *et al.*, 1953). Die vorliegende Arbeit soll als Grundlage für weitere Untersuchungen in dieser Richtung dienen. Sie befasst sich nur mit der Embryonalentwicklung normaler Genotypen und sollte spätere Vergleiche mit letalen Keimen ermöglichen.

* Herrn Professor Dr. E. HADORN bin ich für die Anregung zu dieser Arbeit, sowie für seine zahlreichen Ratschläge zu herzlichem Dank verpflichtet. Herrn Dr. P. S. CHEN danke ich bestens für seine Hilfe in technischen Fragen und bei der Zusammenstellung des Manuskriptes.

METHODE

Aus den Gelegen eines *Drosophila*-Wildstammes (Sevelen) wurden Extrakte hergestellt und diese sowohl ein—als auch zweidimensional chromatographiert. Für ein eindimensionales Chromatogramm genügte ein Extrakt aus 250 Eiern, während 1000 Eier extrahiert werden mussten, um im zweidimensionalen Chromatogramm auch die schwächeren Flecken sichtbar zu machen. Die Eier wurden in zweistündigen Gelegen bei 25° gewonnen. In Anlehnung an ULRICH (1953) wurden kleine Futterschälchen mit reichlich Hefe versehen

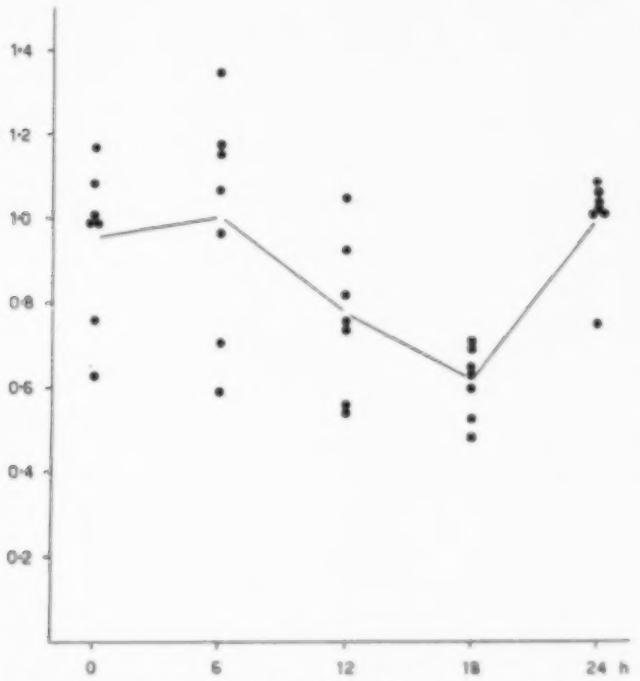


ABB. 1. Gesamtmenge der ninhydrinpositiven Substanzen pro 250 Eier während der Embryonalentwicklung von *Drosophila melanogaster*.
Ordinate: Extinktionseinheiten.
Abszisse: Entwicklungsalter in Stunden (h) nach Eiablage.

und mit einem mit Holtfreter benetzten Filterpapierstreifen belegt. Darauf wurden wiederum kleine Filterpapierstreifchen leiterartig angeordnet. Es bestätigte sich, dass die Fliegen ihre Eier mit Vorliebe an die Kanten dieser Streifchen legten, was das Sammeln sehr erleichterte. Die Eier konnten mit dem Pinsel zusammengewischt und gezählt werden. Da sie nicht mit Hefe verunreinigt waren, genügte es, sie anschliessend noch kurz in destilliertem Wasser zu spühlen. Je 1000 Eier (für ein eindimensionales Chromatogramm 250) wurden in 0,3 ccm 80% Methylalkohol fein zerrieben und anschliessend zentrifugiert. Der Extrakt wurde auf Whatman Filterpapier Nr. 1 aufgetragen, aufsteigend in 70% Propanol und anschliessend absteigend in wassergesättigtem Phenol bei Zimmertemperatur chromatographiert. Das Phenol wurde durch Warmluft (*ca.* 45°) während etwa 2 Stunden aus dem Papier entfernt.

Die quantitative Bestimmung der Aminosäuren geschah nach der abgeänderten Methode von BODE *et al.* (1952). Die Chromatogramme wurden beidseitig mit 0,5% Ninhydrinlösung besprüht und während 30 Minuten bei 80° getrocknet. So erscheinen die Flecken deutlich sichtbar. Anschliessendes Besprühen mit destilliertem Wasser und nochmaliges Spritzen mit 0,5% Ninhydrin entwickelt das Chromatogramm vollständig. Die Flecken wurden nun sogleich planimetriert, ausgeschnitten und in je 5 ccm Methanol, dem 0,1 ccm saure Kupfernitratlösung (1 ccm gesättigte wässerige Lösung von $Cu(NO_3)_2$ +0,1 ccm 10% HNO_3 in 100 ccm Methanol) beigegeben wurde, eluiert. Der rote Kupferkomplex, der dadurch entsteht, ist stabil und die Lösungen können einige Zeit im Eisschrank aufbewahrt werden. Darauf wurden im Beckman-Photospektrometer bei einer Wellenlänge von 510 mμ die Extinktionswerte bestimmt und die Papierwerte von den gemessenen Werten abgezogen (vergl. BENZ, 1955).

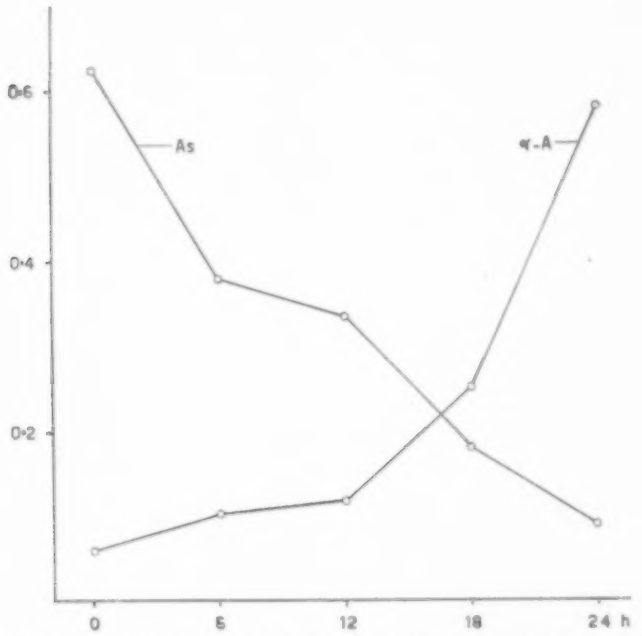


ABB. 2. Mengenveränderung der Asparaginsäure (As) und des α-Alanin (α-A) während der Embryonalentwicklung von *Drosophila melanogaster* (Extrakte aus 1000 Eiern). Jeder Punkt ist ein Mittelwert aus 10–11 Einzelbestimmungen.
Ordinate : Extinktionseinheiten.
Abszisse : Entwicklungsalter in Stunden (h) nach Eiablage.

ERGEBNISSE

Die Eier wurden bei 25°±0,5°C ihrer Entwicklung überlassen und im Alter von 0hr, 6hr, 12hr und 18hr extrahiert, wobei jedoch immer mit einer Streuung des Alters um ± 1hr gerechnet werden muss, da die Eier in 2-stündigen Gelegen gewonnen wurden. Ergänzend wurden auch noch 24hr alte Larven des 1. Stadiums, die noch keine Nahrung zu sich genommen hatten, chromatographiert.

(*a*) *Ein-dimensionale Chromatogramme*

Aus den Mittelwerten einer Serie von je 7 Versuchen geht hervor, dass die Gesamtmenge der ninhydrinpositiven Substanzen unmittelbar vor dem Schlüpfen der Larven ein Minimum erreicht, anschliessend steigt sie wieder an (Abb. 1).

(*b*) *Zwei-dimensionale Chromatagramme*

Eine Zusammenstellung der Aminosäuren und Peptide, die während der Embryonalentwicklung von *Drosophila* auftreten, findet sich in Tabelle 1. β-Alanin ist in 0–18 Stunden alten Eiern höchstens spurenweise vorhanden, tritt aber bei frischgeschlüpften Larven deutlich bestimmbar hervor. Arginin und

TABELLE 1—DIE FREIEN AMINOSÄUREN UND POLYPEPTIDE UND IHRE EXTINKTIONSWERTE* (IN EXTINKTIONSEINHEITEN DES BECKMAN-PHOTOSPEKTROMETER) WÄHREND DER EMBRYONALENTWICKLUNG VON *Drosophila melanogaster*. FÜR JEDE BESTIMMUNG WURDE EIN EXTRAKT AUS 1000 EIERN ODER FRISCHGESCHLÜPFTEN LARVEN VERWENDET

	0 h	6 h	12 h	18 h	24 h
Asparaginsäure	0,626 ± 0,017	0,381 ± 0,019	0,336 ± 0,015	0,179 ± 0,009	0,091 ± 0,016
Glutaminsäure	0,690 ± 0,014	0,845 ± 0,022	0,707 ± 0,014	0,496 ± 0,022	0,357 ± 0,009
Glutamin	0,269 ± 0,014	0,160 ± 0,010	0,262 ± 0,010	0,232 ± 0,013	1,031 ± 0,211
Serin	0,096 ± 0,007	0,124 ± 0,007	0,080 ± 0,004	0,084 ± 0,005	0,226 ± 0,018
Taurin	0,094 ± 0,001	0,081 ± 0,009	0,079 ± 0,007	0,075 ± 0,006	0,074 ± 0,007
Cystin	0,052 ± 0,006	0,041 ± 0,007	?	?	—
Glykokoll	0,126 ± 0,006	0,158 ± 0,008	0,141 ± 0,004	0,092 ± 0,006	0,119 ± 0,008
Threonin	0,031 ± 0,005	0,047 ± 0,005	0,065 ± 0,004	0,059 ± 0,004	0,088 ± 0,011
α-Alanin	0,059 ± 0,005	0,103 ± 0,010	0,118 ± 0,014	0,251 ± 0,035	0,581 ± 0,047
β-Alanin	—	—	—	—	0,071 ± 0,007
Histidin	0,050 ± 0,005	0,054 ± 0,007	0,070 ± 0,004	0,079 ± 0,006	0,089 ± 0,003
Lysin	0,094 ± 0,013	0,054 ± 0,009	0,053 ± 0,006	0,077 ± 0,010	0,083 ± 0,009
Arginin	?	?	?	0,053 ± 0,012	0,054 ± 0,009
Tyrosin	0,036 ± 0,007	0,040 ± 0,004	0,038 ± 0,006	—	—
Tryptophan	0,053 ± 0,005	0,049 ± 0,005	0,031 ± 0,006	0,031 ± 0,005	?
γ-Aminobuttersäure	—	—	—	0,039 ± 0,004	0,054 ± 0,008
P_1	0,065 ± 0,005	0,069 ± 0,010	0,107 ± 0,010	0,084 ± 0,007	0,084 ± 0,010
P_2	0,260 ± 0,017	0,162 ± 0,017	0,121 ± 0,020	0,181 ± 0,019	0,369 ± 0,026
P_3	0,030 ± 0,004	0,032 ± 0,003	0,020 ± 0,004	0,028 ± 0,003	0,031 ± 0,004

* Mittelwerte aus je 10–11 Einzelbestimmungen

γ-Aminobuttersäure treten ebenfalls erst mit Sicherheit nach 18–24 Stunden auf, wogegen Cystin in Laufe der Embryonalentwicklung verschwindet. Prolin, Valin und Leucin, die bei älteren Larven regelmässig festgestellt werden (CHEN und HADORN, 1954; STUMM-ZOLLINGER, 1954; BENZ, 1955), fehlen oder sind nur in Spuren nachzuweisen. Tyrosin und Tryptophan treten unregelmässig auf. Tyrosin fehlt bei 18–24 Stunden alten Eiern oder Larven ganz, Tryptophan ist unsicher.

Die von HADORN und MITCHELL (1951) als "Larvin", von BENZ (1955) als "Larvin I" und "Larvin II", von CHEN und HADORN (1954) und STUMM-ZOLLINGER (1954) als P_1 und P_2 bezeichneten Peptide kommen auch in den Eiern von *Drosophila* vor. P_3 (CHEN und HADORN, 1954) oder Metamorphin (BENZ, 1955) findet sich in allen Eistadien und liegt dicht unter Glykokoll. Das von HADORN

und MITCHELL (1951) beschriebene Frontpeptid wurde auch festgestellt. Seine Konzentration variiert stark, und es ergaben sich für die einzelnen Stadien keine übereinstimmenden Resultate.

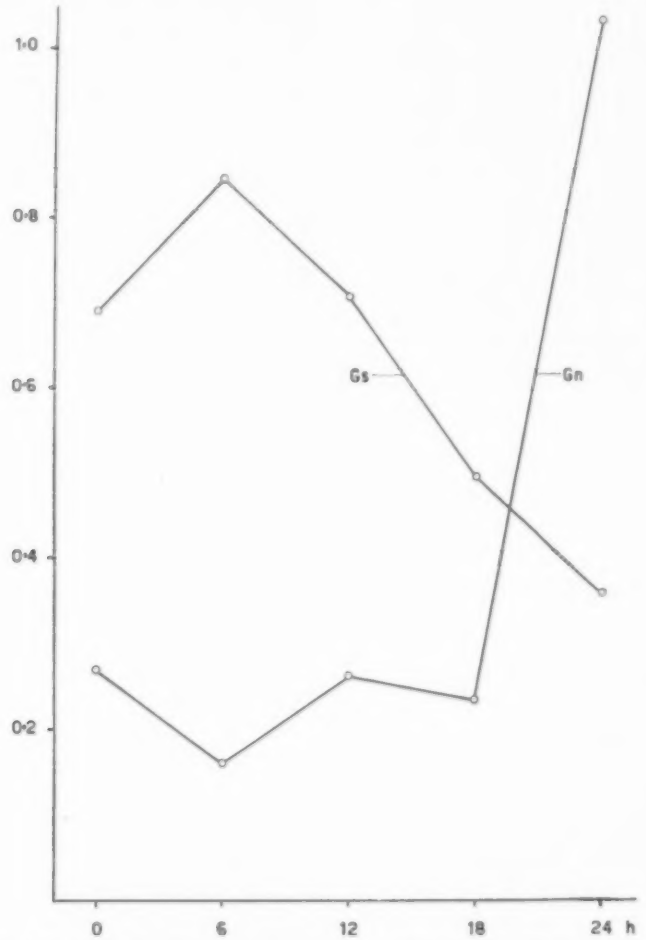


ABB. 3. Mengenveränderung der Glutaminsäure (Gs) und des Glutamin (Gn) während der Embryonalentwicklung von *Drosophila melanogaster* (Extrakte aus 1000 Eiern). Jeder Punkt ist ein Mittelwert aus 10–11 Einzelbestimmungen.
Ordinate : Extinktionseinheiten.
Abszisse : Entwicklungsalter in Stunden (h) nach Eiablage.

Wichtiger zur Charakterisierung der Aminosäuren und Polypeptide während der Embryonalentwicklung sind die quantitativen Bestimmungen (Tabelle 1). Freie Asparaginsäure ist in frischabgelegten Eiern in grosser Menge vorhanden, verschwindet dann aber zum grössten Teil. α-Alanin verstärkt sich während der

ganzen Embryonalentwicklung stetig (Abb. 2). Glutaminsäure nimmt nach einer vorübergehenden Verstärkung (6 Stunden) stark ab. Glutamin dagegen erreicht nach 6 Stunden ein Minimum und nimmt zwischen 18 und 24 Stunden, also während und gleich nach dem Schlüpfen, um das drei- bis vierfache zu (Abb. 3). Die Konzentrationsschwankungen der übrigen Stoffe sind wesentlich geringer. Bei Threonin und Histidin kann eine leichte Verstärkung, bei Taurin ein schwacher Abfall festgestellt werden. Glykokoll ist in Eiern von 6 Stunden etwas intensiver, wird aber nachher wieder reduziert. Serin ist in frisch geschlüpften Larven deutlich verstärkt. P_1 erreicht ein Maximum nach 12 Stunden, wogegen P_2 im selben Zeitpunkt ein Minimum aufweist.

SCHLUSSBEMERKUNG

Es ist anzunehmen, dass während der Embryonalentwicklung die vorhandenen Dotterproteine zu freien Aminosäuren und einfachen Peptiden abgebaut werden, um die Synthese von spezifischen Gewebeproteinen zu ermöglichen (KAVANAU, 1954). Nach intensivem Abbau ist die Konzentration der freien Aminosäuren hoch, nach Vollzug der Synthese dagegen gering.

Da Asparaginsäure, Glutaminsäure, Glutamin und α-Alanin am stärksten vertreten sind und die grössten quantitativen Schwankungen zeigen, scheinen sie während der Embryonalentwicklung von *Drosophila melanogaster* eine besondere Rolle zu spielen. In die gleiche Richtung weisen auch die Befunde von NAKAMURA, IMAIZUMI und TAKANAMI (1952) für die Embryonalentwicklung von *Drosophila virilis*. Asparaginsäure, Glutaminsäure und Glutamin sind wichtige Ausgangsmaterialien für die Eiweissynthese. Im Glutamin können Aminogruppen gespeichert und von da aus für den Aufbau anderer Aminosäuren benützt werden. Die Kurve des Glutamin verläuft entgegengesetzt zu derjenigen der Glutaminsäure und der Asparaginsäure (vergl. Abb. 2 und 3). CHEN (1956) konnte dasselbe bei Urodelenembryonen festellen. Das α-Alanin ist keine essentielle Aminosäure und kann durch Transaminierung (LANG, 1952, S. 203) und direkt aus Kohlehydraten (BALDWIN, 1952, S. 449) gebildet werden.

Die Bestimmung der freien Aminosäuren kann nur einige erste Anhaltspunkte zur Deutung des komplizierten Proteinstoffwechsels während der Embryonalentwicklung geben.

LITERATUR

BALDWIN E. (1952) *Dynamic aspects of biochemistry*. Cambridge.

BENZ G. (1955) Quantitative Veränderungen der Aminosäuren und Polypeptide während der Entwicklung von *Drosophila melanogaster*. *Arch. Klaus-Stift. Vererbforsch.* **30**, 498.

BODE F. H., HÜBNER H. J., BRÜCKNER H., und HOERES K. (1952) Eine einfache quantitative Bestimmung von Aminosäuren im Papierchromatogramm. *Naturwiss.* **39**, 525.

BOELL E. J. and POULSON D. F. (1939) The respiratory metabolism of normal and genetically deficient eggs of *Drosophila melanogaster*. *Anat. Rec.* **75** (Suppl.), 65.

BULL A. L. (1954) A study of the oxygen consumption of normal and lethal embryos of *Drosophila melanogaster*. *Z. indukt. Abstamm.- u. Vererb. Lehre* **86**, 134.

VOL. 3 1959

CHEN P. S. (1956) Metabolic changes in free amino acids and peptides during urodele development. *Exp. Cell. Res.* **10**, 675.

CHEN P. S. und HADORN E. (1954) Vergl. Untersuchungen über die freien Aminosäuren in der larvalen Haemolymphe von *Drosophila, Ephestia* und *Corethra*. *Rev. suisse Zool.* **61**, 437.

HADORN E. (1955) *Letalfaktoren*. G. Thieme, Stuttgart.

HADORN E. and MITCHELL H. K. (1951) Properties of mutants of *Drosophila melanogaster* and changes during development as revealed by paper chromatography. *Proc. nat. Acad. Sci., Wash.* **37**, 650.

KAVANAU J. L. (1954) Amino acid metabolism in developing sea urchin embryos. *Exp. Cell. Res.* **6**, 563.

LANG K. (1952) *Der intermediäre Stoffwechsel*. Springer, Berlin.

NAKAMURA K., IMAIZUMI T., and KITAZUME Y. (1951a) Amino acids in *Drosophila melanogaster*. *Drosophila Inform. Serv.* **25**, 120.

NAKAMURA K., KITAZUME Y., TAKANAMI M., and IMAIZUMI T. (1951b) Free amino acids in different developmental stages and constituent amino acids of eggs of *Drosophila*. *Pap. Co-ord. Commit. Res. Gen.* **2**, 163.

NAKAMURA, K., IMAIZUMI T., and TAKANAMI M. (1952) Changes in amino acids during the early development of *Drosophila virilis*. *Drosophila Inform. Serv.* **26**, 114.

NAKAMURA K., IMAIZUMI T., KITAZUME Y., SHIOMI T., and TAKANAMI M. (1953) Biochemical studies of embryonic lethal factors in two strains of *Drosophila melanogaster*. *Drosophila Inform. Serv.* **27**, 108.

STUMM-ZOLLINGER E. (1954) Vergleichende Analyse der Aminosäuren und Peptide in der Haemolymphe des Wildtyps und der Mutante "*letal-translucida*" (*ltr*) von *Drosophila melanogaster*. *Z. indukt. Abstamm.- u. Vererb. Lehre* **86**, 126.

ULRICH H. (1953) A convenient method of collecting large numbers of *Drosophila* eggs homogeneous in age. *Drosophila Inform. Serv.* **27**, 124.

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 57 *to* 62. *Pergamon Press Ltd., London. Printed in Great Britain*

AMINO ACID OXIDASE ACTIVITY IN THE FAT BODY AND MALPIGHIAN TUBES OF SOME INSECTS*

JACQUES L. AUCLAIR†

Department of Entomology, Cornell University, Ithaca, N.Y.

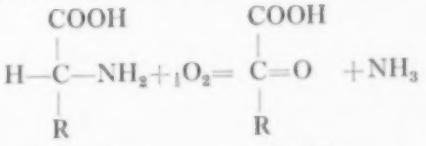
(*Received* 12 *June* 1958)

Abstract—By incubating the fat body or the Malpighian tubes in a saline-buffer amino acid mixture, α-keto acids were produced from a number of *D*- and *DL*-amino acids. These results suggest the presence of a *D*-amino acid oxidase in the fat body and Malpighian tubes of the American cockroach, *Periplaneta americana* (L.), and the German cockroach, *Blattella germanica* (L.), and in the fat body of the greater wax moth, *Galleria mellonella* (L.). Other tissues from these insects as well as the fat body and Malpighian tubes of the large milkweed bug, *Oncopeltus fasciatus* (Dall.), gave negative results.

VOL. 3 1959

INTRODUCTION

THE proteins of the food are hydrolysed in the digestive tract of mammals to free amino acids which diffuse through the digestive wall into the blood. Some of the absorbed amino acids passing through the liver and kidneys are deaminated. Deamination may be performed as a preliminary step to transamination, or to provide the necessary substrate for carbohydrate and lipid syntheses. Enzymes which catalyse deamination include *D*-amino acid oxidase, *L*-amino acid oxidase, *L*-glutamic dehydrogenase, and glycine oxidase. They are apparently widely distributed but their concentration in mammals is high in the liver and kidneys. One of the earliest experimental studies on deamination was made by KREBS (1933, 1935) who discovered the *D*- and the *L*-amino acid oxidase activity of pig kidney, using the tissue slice technique. Enzymatic transamination was apparently first reported by BRAUNSTEIN and KRITZMANN (1937a, b). According to BALDWIN (1957), deamination with production of ammonia may be accomplished by two processes, hydrolytic and oxidative. However, very little experimental evidence supports the hydrolytic process in animal metabolism, the bulk of information being in favour of oxidative deamination, which is achieved as follows :



α-Amino Acid α-Keto Acid

This reaction would be essentially the same for *D*- and *L*-amino acids.

* This investigation was aided by a grant from the Lalor Foundation, Wilmington, Delaware. The material presented herewith was part of a doctorate thesis submitted to the Graduate School, Cornell University, 1949.

† Entomologist, Science Service Laboratory, St. Jean, Quebec, Canada.

In insects, knowledge of the intermediary metabolism of amino acids and more particularly of the site and nature of deamination is very limited. BROWN and FARBER (1936) detected deaminase activity in homogenates of larvae of *Lucilia sericata* Mg. and *Calliphora erythrocephala* Mg. The enzyme was active on higher protein breakdown products but inactive on amino acids and simple peptides. Similar results were obtained by LENNOX (1941) working with homogenates of larvae of *Lucilia cuprina* (Wiedemann). BARRON and TAHMISIAN (1948) working on *Periplaneta americana* (L.) reported that the alanine–glutamate transamination reaction is catalysed *in vitro* by cockroach muscle homogenate. AGRELL (1949) observed that methylene blue was reduced more rapidly by homogenates of pupae of *Calliphora erythrocephala* Mg. when glutamate, alanine, glycine, or valine was added. A brief review by AUCLAIR (1953) summarized some recent investigations on amino acids in insect blood, on their metabolism and importance in insect nutrition. This is a report on amino acid oxidase activity in the fat body and Malpighian tubes of a few insect species. After the completion of the present work, BHEEMESWAR and SREENIVASAYA (1952) presented evidence that the haemolymph and the glandular and the intestinal tracts of the silkworm, *Bombyx mori* (L.), contain a transaminase capable of mediating the transfer of the amino group of aspartic acid to α-ketoglutaric acid. KILBY and NEVILLE (1956, 1957) working on the desert locust, *Schistocerca gregaria* Forsk., reported that (1) homogenates of fat body catalyse transamination reactions between α-ketoglutarate and several α-amino acids, (2) fat body, Malpighian tubes, and mid-gut wall homogenates are able to convert glutamic acid into glutamine, and (3) fat body contains arginase, *D*-amino acid oxidase, a less active *L*-amino acid oxidase, and a glutamate-cytochrome *c* reductase system.

MATERIALS AND METHODS

The experimental insects were reared under standardized laboratory conditions, and the species with the respective diets used are given in Table 1.

Tissues such as fore-, mid-, and hind-gut, salivary glands, Malpighian tubes, gastric caeca, fat body, muscles, male accessory glands, and oöcytes, were dissected out of the living insect and rinsed in several changes of physiological saline. They were ground into a homogenate in a glass micro-mortar or used *in toto* and mixed with a substrate of one or several *D*-, *L*-, or *DL*-amino acids, $\frac{1}{15}$ molar in a saline-buffer solution of *p*H 8·2, the saline being prepared according to YEAGER (1939). A droplet of toluene was added to the preparations. Control preparations were also made without amino acids, saline, tissues, or toluene. Incubation was carried out at 37°C for 3–6 hr. The method for measuring amino acid oxidase activity made use of the reagent 2,4,dinitrophenylhydrazine (DPH), as described by SEALOCK (1941) and HOROWITZ (1944). This method has been used satisfactorily by KREBS (BALDWIN, 1957), GREEN *et al.* (1943), and CEDRANGOLO (1947) for the study of *D*- and *L*-amino acid oxidase activity in crude enzyme preparations and tissue incubations. In acid solution, DPH reacts with the α-keto acids produced by the oxidative deamination of amino acids to give the corresponding

2,4,dinitrophenylhydrazones. If an excess of 2N NaOH is then added, a purple colour appears which is characteristic of these hydrazones. The colour intensity can be determined in a colorimeter against a standard for quantitative purposes. In the present work, the incubations and the DPH tests were made in small

TABLE 1—TESTS INSECTS AND DIET

Species	Diet
Adults	
Blattella germanica (L.) (German cockroach)	Whole wheat flour 40 per cent Dried skim milk 40 per cent Dried brewer's yeast 20 per cent Water
Periplaneta americana (L.) (American cockroach)	Dog food (Big Red, G.L.F. Corp.) protein 25 per cent fat 4 per cent fibre 5 per cent Water
Oncopeltus fasciatus (Dall.) (Milkweed bug)	Milkweed seeds Water
Larvae	
Galleria mellonella (L.) (Greater wax moth)	Pablum moistened with honey, glycerine and water

biological culture tube caps fitted in test tube holders in which each cell receiving a tube was lined with white paper. This simple device, functioning in a manner similar to the Dubosq colorimeter, increased the sensitivity of the DPH test. Slight differences in the colour intensities between solutions of the incubates could be detected, and semi-quantitative comparisons were possible.

RESULTS AND DISCUSSION

The mean results obtained from about 400 series of incubations are summarized in Tables 2 and 3. α-keto acids were produced when a number of *D*- or *DL*-amino acids were incubated with the fat body or the Malpighian tubes of *P. americana* and *B. germanica*, or with the fat body of *G. mellonella*. Other tissues from these insects as well as the fat body and Malpighian tubes of *O. fasciatus* gave negative results. These results indicate that the deaminase activity of the fat body and Malpighian tubes is mainly caused by a *D*-amino acid oxidase. The function and importance of this enzyme in the intermediary metabolism of mammals is not well understood since naturally occurring amino acids are mostly of the *L* form. One of the possible functions for this enzyme would be to destroy those amino acids that may be formed by the racemization of the natural amino acids (SUMNER and SOMERS, 1947). In considering this suggestion, it may be noted that the milkweed bug tissues failed to show any deaminase activity (Table 3), and it is in the blood of that species that the presence of an unnatural isomer, *D*-alanine, was reported (AUCLAIR and PATTON, 1950).

BALDWIN (1957) mentioned that *D*-amino acid oxidase does not attack glycine, *D*-glutamic acid, and *D*-lysine. Furthermore, arginine and ornithine might never

undergo deamination in living tissues. The amino acid oxidase of the fat body of the locust, as reported by KILBY and NEVILLE (1957), acted upon *DL*-alanine, *L*-alanine, *DL*-valine, *L*-valine, *DL*-leucine, *L*-leucine, *DL*-phenylalanine, and

TABLE 2—AMINO ACID OXIDASE ACTIVITY OF FAT BODY AND MALPIGHIAN TUBES OF *P. americana* (L.) AS DETERMINED BY THE RELATIVE CONCENTRATION OF α-KETO ACIDS AFTER INCUBATION WITH AMINO ACIDS

Rapidly oxidized	Very slowly oxidized	Not oxidized
By fat body	By both tissues	By both tissues
D-Alanine	*DL*-Serine	β-Alanine
DL-Alanine	*DL*-Threonine	*D*-Arginine HCl
DL-α-Amino-butyric acid	*DL*-Tryptophan	*L*-Arginine HCl
D-Methionine	By fat body	*L*-Asparagine
DL-Methionine	*DL*-Homocystine	*L*-Cysteine HCl
D-Norleucine	*DL*-*Iso*leucine	*L*-Cystine
DL-Norleucine	*L*-Leucine	*D*-Glutamic acid
DL-Norvaline	*DL*-Proline	*L*-Glutamic acid
Slowly oxidized	By Malpighian tubes	*L*-Glutamine
By fat body	*D*-Alanine	Glycine
DL-Leucine	*DL*-Alanine	*DL*-Histidine
DL-Phenylalanine	*DL*-Leucine	*L*-Hydroxyproline
D-Serine	*DL*-Norvaline	*L*-Lysine HCl
D-Threonine	*DL*-Phenylalanine	*DL*-Lysine HCl
By Malpighian tubes	*D*-Serine	*DL*-Ornithine HCl
DL-α-Amino-butyric acid	*D*-Threonine	*L*-Proline
DL-Aspartic acid	*DL*-Valine	*L*-Taurine
D-Methionine		*L*-Tryptophan
DL-Methionine		*L*-Tyrosine
D-Norleucine		By fat body
DL-Norleucine		*DL*-Aspartic acid
		DL-Valine
		By Malpighian tubes
		DL-Homocystine
		DL-*Iso*leucine
		L-Leucine
		DL-Proline

L-phenylalanine. These results are in close agreement with those reported in Table 2 for *D*-amino acid oxidase.

A slight deaminase activity was observed when the fat body of *P. americana* was incubated with *L*-leucine (Table 2), and doubtful results were obtained with the fat body of *G. mellonella* (Table 3), indicating the possible action of a weak *L*-amino acid oxidase. Incubations with a few other *L*-amino acids as substrates gave very weak positive results, but these could not always be repeated. It is probable, however, that fat body contains a weak *L*-amino acid oxidase and that the activity of this enzyme was inhibited under the conditions of the experiments.

This enzyme in mammals is much less active and more easily denatured than the *D*-amino acid oxidase. Results by KILBY and NEVILLE (1957) indicated that the fat body of *S. gregaria* contained an active *D*-amino acid oxidase together with a less active *L*-amino acid oxidase.

TABLE 3—AMINO ACID OXIDASE ACTIVITY OF FAT BODY AND MALPIGHIAN TUBES FROM THREE INSECT SPECIES

Substrate	Wax moth larvae		German cockroach adults		Milkweed bug adults	
	M. tubes	F. body	M. tubes	F. body	M. tubes	F. body
D-Methionine	—	+	+	?	—	—
DL-Methionine	—	+	+	+	—	—
DL-Norleucine	—	+	+	—	—	—
L-Leucine	—	?	—	—	—	—

VOL. 3 1959

When either the fat body or Malpighian tubes was homogenized before incubation, or when toluene was added, the production of α-keto acids was considerably decreased as compared to that obtained with *in toto* tissues or with incubates without toluene. The presence of toluene in the incubates did not interfere with the DPH test. Toluene is recommended as an antiseptic when studying enzymatic reactions, but it may have an unfavourable effect upon enzyme action (SUMNER and SOMERS, 1947). Incubations without toluene carried out with tissues other than fat body and Malpighian tubes always gave negative results, ruling out therefore the possibility of external contamination by micro-organisms responsible for the observed deaminase activity when fat body or Malpighian tubes were used. It is indeed very doubful whether external contamination could have produced any effect during the short incubation period used. However, the possibility that micro-organisms already present in the Malpighian tubes or the fat body were responsible for the production of α-keto acids cannot be ruled out. STEINHAUS (1947) gives a long list of bacteria (e.g. *Bacillus* sp., *Micrococcus* sp.) and protozoa (e.g. Ciliata, Sporozoa, Flagellates) normally found in the alimentary canal of *P. americana*. It is possible that many of these micro-organisms eventually find their way into the Malpighian tubes. The sporozoan *Plistophora periplanetae* for instance, although observed only in the alimentary tract of *P. americana* has been observed in the Malpighian tubes of other cockroaches such as *B. germanica* and *B. orientalis*. Intracellular symbionts exist in the fat body of *P. americana* (e.g. GIER, 1936) and *B. germanica* (STEINHAUS, 1947). Whether the production of α-keto acids is due to an enzyme secreted by these intracellular symbionts or secreted by cells of the fat body does not change the end results. Intracellular symbionts are considered as a physiological part of the cockroach; they are apparently always present, being transmitted from one generation to the next through the egg. The use of aposymbiotic roaches (RICHARDS and BROOKS, 1958) might throw some light on the above problem.

Acknowledgement—The writer wishes to express his sincere gratitude and appreciation to the project leader, Dr. R. L. PATTON, Professor of Insect Physiology at Cornell University, for his encouragement and assistance throughout this work and to the Lalor Foundation of Wilmington, Delaware, for financial support in the project.

REFERENCES

AGRELL I. (1949) Occurrence and metabolism of free amino acids during insect metamorphosis. *Acta physiol. scand.* **18**, 247–258.

AUCLAIR J. L. (1953) Amino acids in insects. *Canad. Ent.* **85**, 63–68.

AUCLAIR J. L. and PATTON R. L. (1950) On the occurrence of *D*-alanine in the haemolymph of the milkweed bug, *Oncopeltus fasciatus*. *Rev. Canad. Biol.* **9**, 3–8.

BALDWIN E. (1957) *Dynamic aspects of biochemistry*. Cambridge Univ. Press, London.

BARRON E. S. G. and TAHMISIAN T. N. (1948) The metabolism of cockroach muscle. *J. cell. comp. Physiol.* **32**, 57–76.

BHEEMESWAR B. and SREENIVASAYA M. (1952) Occurrence of transaminase in the silkworm, *Bombyx mori* (L.). *Curr. Sci.* **21**, 253–255.

BRAUNSTEIN A. E. and KRITZMANN M. G. (1937a) Über den Ab- und Aufbau von Aminosäuren durch Umaminierung. *Enzymologia* **2**, 129–137.

BRAUNSTEIN A. E. and KRITZMANN M. G. (1937b) Die Gleichgewichtsreaktion zwischen 1 (+) Glutaminsäure und Brenztraubensäure, bzw. 1 (+) Alanin und α-Ketoglutarsäure. *Enzymologia* **2**, 138–146.

BROWN A. W. A. and FARBER L. (1936) On the deaminating enzyme of flesh-fly larvae. *Biochem. J.* **30**, 1107–1118.

CEDRANGOLO F. (1947) Sulla Formazione dell' α-chetoacido dal respettivo L-aminoacido. *Enzymologia* **12**, 151–155.

GIER H. T. (1936) The morphology and behaviour of the intracellular bacteroids of roaches. *Biol. Bull., Woods Hole* **71**, 433–452.

GREEN D. E., NOCITO V., and RATNER S. (1943) *L*-Amino acid oxidase of animal tissues. *J. biol. Chem.* **148**, 461–462.

HOROWITZ N. H. (1944) The *D*-amino acid oxidase of *Neurospora*. *J. biol. Chem.* **154**, 141–149.

KILBY B. A. and NEVILLE E. (1956) Amino acid metabolism in locust tissues. *Biochem. biophys. Acta* **19**, 389–390.

KILBY B. A. and NEVILLE E. (1957) Amino acid metabolism in locust tissues. *J. Exp. Biol.* **34**, 276–289.

KREBS H. A. (1933) Untersuchungen über den Stoffwechsel der Aminosäuren in Tierkorper. *Hoppe-Seyl. Z.* **217**, 191–194.

KREBS H. A. (1935) Metabolism of amino acids. III. Deamination of amino acids. *Biochem. J.* **29**, 1620–1644.

LENNOX F. G. (1941) Studies on the physiology and toxicology of blowflies. 9. The enzymes responsible for ammonia production by larvae of *Lucilia cuprina*. *Pamphl. Coun. sci. industr. Res. Aust.* **5** (109), 37–64.

RICHARDS A. G. and BROOKS M. A. (1958) Internal symbiosis in insects. *Ann. Rev. Ent.* **3**, 37–56.

SEALOCK R. R. (1941) The determination of amino acids of the dextro or unnatural configuration. *Science* **94**, 73–74.

SUMNER J. B. and SOMERS G. F. (1947) *Chemistry and methods of enzymes*. Academic Press Inc., New York.

STEINHAUS E. A. (1947) *Insect microbiology*. Comstock Publ. Co., Ithaca, N.Y.

YEAGER J. F. (1939) Electrical stimulation of isolated heart preparations from *Periplaneta americana*. *J. agric. Res.* **59**, 122–123.

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 63 *to* 73. *Pergamon Press Ltd., London. Printed in Great Britain*

PTERIDINES IN THE MILKWEED BUG, *ONCOPELTUS FASCIATUS* (DALLAS)—II

QUANTITATIVE DETERMINATION OF PTERIDINE CONTENT OF TISSUES DURING GROWTH*

BRUCE W. HUDSON,† ALLEN H. BARTEL,‡ and RODERICK CRAIG

Department of Entomology and Parasitology, University of California, Berkeley

(*Received* 12 *June* 1958)

VOL. 3 1959

Abstract—A method for the quantitative analysis of xanthopterin, *iso*xanthopterin, and 2-amino-4-hydroxypteridine in the milkweed bug, *Oncopeltus fasciatus* (Dallas), is described. Changes in amounts of these pteridines during growth and development and starvation are given, and the relative amount of nitrogen excreted as pteridine and uric acid has been calculated.

INTRODUCTION

We have reported (Bartel, Hudson, and Craig, 1958) on the kinds of pteridines present in the milkweed bug, *Oncopeltus fasciatus* (Dallas), and their distribution within this insect. In order to study the metabolism of these substances further it was necessary to develop quantitative analytical methods for the pteridines. In this report we present these methods and a brief study of the pteridine content of the tissues of *Oncopeltus* during growth and development.

There is no known quantitative chemical method of analysis specific for pteridines present in biological extracts. The obvious approach is to isolate the pteridines in as pure a form as possible followed by determination by physical methods. Isolation by both ion exchange and paper chromatography was investigated. Paper chromatography gave adequate separations and was much faster. Because of the very high absorption of ultra-violet light by the pteridines, spectrophotometry seemed the method of choice. We found, however, that fluorescence is more sensitive and can be used with the pteridines *in situ* on the filter paper. The use of fluorescence makes complete isolation from most other substances unnecessary, since only those substances that interfere with pteridine fluorescence must be removed. The determination of the pteridines present in *Oncopeltus* by fluorescence of the spot on paper chromatograms is both qualitative and quantitative and permits several pteridines to be determined on the same chromatogram.

* This work was supported in part by funds from the office of the Surgeon General, U.S. Army.

† Present address: Allergy Department, Kaiser Foundation Hospital, San Francisco, California.

‡ Present address: Dept. of Biology, University of Houston, Houston, Texas.

METHODS

Oncopeltus colonies were reared at 28°C on a diet of milkweed seeds and water. Synthetic pteridines used for comparative purposes and analytical standards are given in Table 1.

TABLE 1—PHYSICAL DATA ON PTERIDINE STANDARDS USED FOR QUANTITATIVE ANALYSIS

Pteridine	Spectrography in water		
	λ max. (mμ)	log ε (mol) max.	Source*
Xanthopterin (H_2O)	255 392	4·26 3·84	ALBERT and WOOD (1952)
*Iso*xanthopterin	254 339	4·06 4·15	ALBERT and WOOD (1953)
2-amino-4-hydroxypteridine	251·5 360	3·19 2·71	American Cyanamid Co. Res. Div. Lot 5167

* We gratefully acknowledge the gifts of these samples by A. ALBERT and by American Cyanamid Company.

(1) *Pteridines*

One-dimensional descending chromatography was used for the isolation of pteridines. The most successful solvent systems used were *n*-butanol : acetic acid (7:3) and *sec*-butanol: formic acid: water (8:2:5). Chromatograms were developed on $\frac{1}{2}$ in. wide strips of Whatman No. 1 filter paper for 12–14 hr at room temperature. After chromatographic development the chromatograms were thoroughly air-dried at room temperature for 1–2 hr and placed in the scanning chamber of a fluorescence measuring apparatus, constructed in this laboratory.† This apparatus consists of: (a) the light source and monochromator of a Beckman DU Quartz Spectrophotometer, (b) a scanning chamber containing a drive mechanism for advancing the paper strip and appropriate filters for elimination of undesirable light, (c) a 1P28 photo multiplier tube and power supply for detection of the fluorescent light, (d) a high impedance D.C. amplifier for amplification of phototube current, and (e) a recording volt-ammeter the chart drive of which is coupled to the paper strip drive mechanism of the scanning chamber.

After appropriate light filters and monochromator settings had been selected, the paper strip and chart drive mechanism was engaged and a recording taken of phototube current. Peaks of phototube current corresponded with peaks of fluorescence on the paper chromatograms (Fig. 3, a). The areas of these peaks above the current baseline (determined by inspection) were then measured with a planimeter.

Incident light used for excitation of fluorescence was 283 mμ light from the Beckman DU Quartz Spectrophotometer. Amounts of fluorescence were assumed to be directly proportional to areas of current peaks recorded by the recording

† Available from Microchemical Specialties Company, 1834 University Avenue, Berkeley.

volt-ammeter. Amounts of pteridines in samples were determined by comparison of amounts of fluorescence (i.e. areas of current peaks) with amounts of fluorescence caused by known quantities of synthetic pteridines.

During the course of the work it was found that xanthopterin and *iso*xanthopterin could not be completely separated by one dimensional chromatography with the solvent systems used. Since the fluorescent colours of these two compounds are different, it is possible to decrease the interference of one with the other by

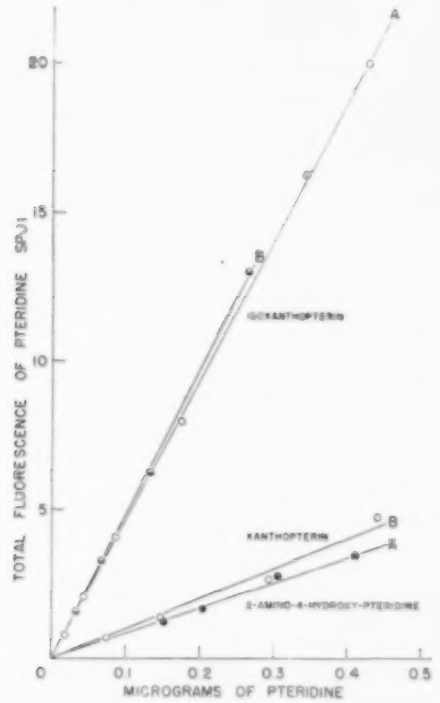


FIG. 1. Standard curves for analysis of pteridines. Total fluorescence is given in terms of area under peaks of curves obtained by fluorescent scanning of chromatograms.
(A) Chromatograms developed with *sec*-butanol : formic acid : water.
(B) Chromatograms developed with *n*-butanol : 5 N acetic acid.

use of a suitable filter. The most useful filter available was the Wratten K_1 gelatin filter. This filter passes approximately 70 per cent and 14 per cent of the emitted fluorescent light of xanthopterin and *iso*xanthopterin respectively. All determinations of xanthopterin were carried out using this filter, the recorded curve being smoothed by eye to correct for the remaining small hump due to *iso*xanthopterin fluorescence.

Concentrated standard solutions containing known amounts of each synthetic pteridine were prepared in 0·1 N NaOH. These solutions were diluted with

0·1 N NaOH to give a series of dilutions for each pteridine. Aliquots of 3·0 μl were chromatographed in triplicate using appropriate chromatographic solvent

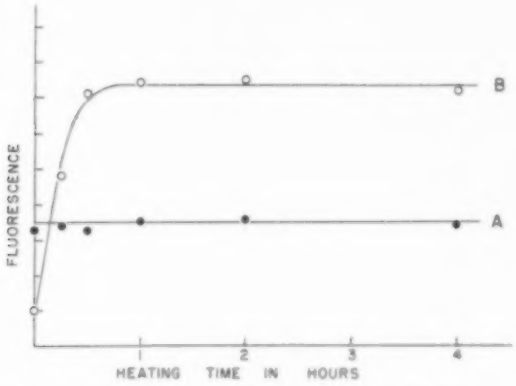


FIG. 2. Effect of time of heating at 100° C in 0·1 N NaOH on measurable fluorescence of A, synthetic xanthopterin, B, hot water extracts of *Oncopeltus*.

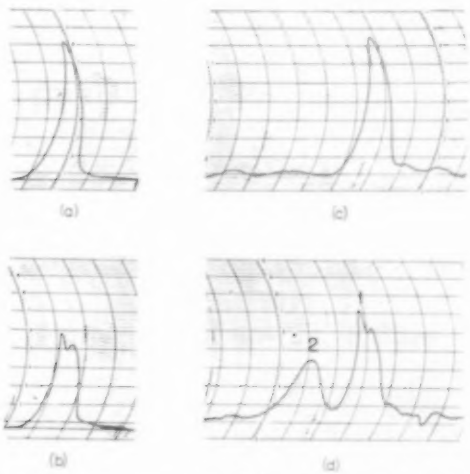


FIG. 3. Fluorescent scannings of chromatograms of pteridines.
(a) Synthetic *iso*xanthopterin.
(b) Synthetic *iso*xanthopterin chromatographed after heating to 100° C in 0·1 N NaOH for 2 hr.
(c) *Oncopeltus* extract.
(d) *Oncopeltus* extract chromatographed after heating to 100° C in 0·1 N NaOH for 2 hr.
1. Double *iso*xanthopterin spot.
2. Xanthopterin.

systems. Values for each determination were averaged and values so obtained were used to construct standard curves (Fig. 1).

Materials to be analysed were homogenized and extracted 3 times in hot distilled water at 100°C. The combined extracts were divided into 2 portions. One portion was used without further treatment for the analysis of *iso*xanthopterin and 2-amino-4-hydroxypteridine by chromatography of 3·0 μl aliquots in the *sec*-butanol:formic acid solvent system followed by measurement of fluorescence. The remaining portion was made 0·1 N with respect to NaOH, sealed in a glass ampoule, heated for 1–2 hr at 100°C, and used for the analysis of xanthopterin after chromatography of 3·0 μl aliquots in the *n*-butanol: 5 N acetic acid solvent system followed by measurement of fluorescence. The hot alkaline treatment is necessary since maximum values of xanthopterin fluorescence were obtained after extracts had been heated for 1 hr in 0·1 N NaOH at 100°C (Fig. 2). The

TABLE 2—RECOVERIES OF PTERIDINES ADDED TO HOT WATER EXTRACTS OF *Oncopeltus*

Pteridine added	Amount present (μg)	Amount added (μg)	Total recovered (μg)	Per cent recovery
Xanthopterin to extract of *Oncopeltus* adults	377	554	900	98
	262	554	781	94
	134	554	651	96
	377	277	629	91
	262	277	528	96
	134	277	401	93
				Average 94·7
*Iso*xanthopterin to extract of *Oncopeltus* adults	104	55	153	91
	104	110	205	92
	104	220	317	96
	52	55	100	94
	52	110	155	94
	52	220	273	100
				Average 94·5
2-amino-4-hydroxypteridine to extract of midgut tissue	2·55	10·1	12·3	97
	5·10	10·1	14·4	92
	10·2	10·1	19·7	94
				Average 94·3

alkaline treatment caused no significant decrease in the measurable fluorescence of synthetic xanthopterin samples but produced erratic results in the amounts of measurable *iso*xanthopterin fluorescence in homogenate extracts and in solutions of synthetic material. These erratic results were presumably due to the development of double spots on the chromatograms (Fig. 3). The measurable fluorescence of 2-amino-4-hydroxypteridine in both the extracts and the synthetic samples showed a steady decrease when heated in 0·1 N NaOH solution. Apparently only xanthopterin is stable in hot 0·1 N NaOH solutions. Recoveries for the analysis of pteridines (see Table 2) averaged 94–95 per cent of pteridine added to hot water extracts of whole *Oncopeltus* or midgut tissue homogenates.

VOL. 3 1959

(2) *Uric acid*

Uric acid was measured by ultra-violet densitometry according to the procedure of BARTEL (1954), see also (MCDONALD, 1955). The technique involves chromatography of materials containing uric acid, measurement of total ultra-violet light absorption by the uric acid spot, and comparison of this absorption with a series of uric acid standards chromatographed and measured in the same manner. Absorption of the uric acid spot was measured with the automatic scanning apparatus previously described. No filter was used. Wave length of light used was 290 mμ. Chromatography was performed using the *n*-butanol:5 N acetic acid system as in the analysis of pteridines.

MATERIALS

Oncopeltus eggs were collected at 24 hr intervals and held at 28°C. Separate analyses were made of eggs after development had proceeded for 0–1, 1–2, 3–4, and 4–5 days. At 4–5 days, 50–60 per cent of the eggs had hatched and this sample consisted of those eggs remaining after removal of hatched individuals.

Larvae of the second through fifth instars and adults of both sexes were chosen at random from a colony of *Oncopeltus* reared at 28°C.

The following tissues and body parts of adult *Oncopeltus* were extracted and analysed: heads, midguts, hindguts and Malpighian tubules, gonads, fat body, abdominal hypodermis, and thorax and included organs (with exception of gut and fat body).

Midguts were analysed after starvation and subsequent feeding. A large sample of *Oncopeltus* adults were selected at random. This sample was separated into two parts and placed in similar 1 gallon, wide mouth jars containing similar water supplies and other materials. After a starvation period of 1 week the midgut tissues of a number of individuals from each colony were analysed. Fresh food material (milkweed seed) was added to one colony and the midguts of individuals in both colonies were analysed at intervals of 1, 2, 4, and 8 days. For each analysis, the midguts of 20–25 individuals were pooled, homogenized and extracted, and the pteridine content measured.

For the collection of excrement, larvae and adults of *Oncopeltus* were kept for a period of 2 weeks in separate 1 gallon, wide mouthed jars. Excrement was collected at the end of this period by scraping deposits from the sides of the jars. Excrement samples were dried thoroughly over $CaCl_2$ under a pressure of 4 mm Hg for a period of 1 week. After grinding and thorough mixing, weighed amounts of the excrement were extracted in hot water and amounts of pteridine and uric acid were measured.

RESULTS AND DISCUSSION

The pteridines of *Oncopeltus* have previously been shown to consist of 2-amino-4-hydroxypteridine, *iso*xanthopterin, and xanthopterin (BARTEL *et al.*, 1958). Xanthopterin usually appears in chromatograms only after initial exposure of the materials to strong acid, base, or after prolonged heating. Xanthopterin in

Oncopeltus is apparently bound or combined with other substances in the natural state, or may appear after the breakdown of a more complex pteridine. The precursor of xanthopterin has been shown to have an R_f=0 in three acidic chromatographic solvent systems (BARTEL *et al.*, 1958). All references to xanthopterin refer to xanthopterin detectable after hot alkaline treatment.

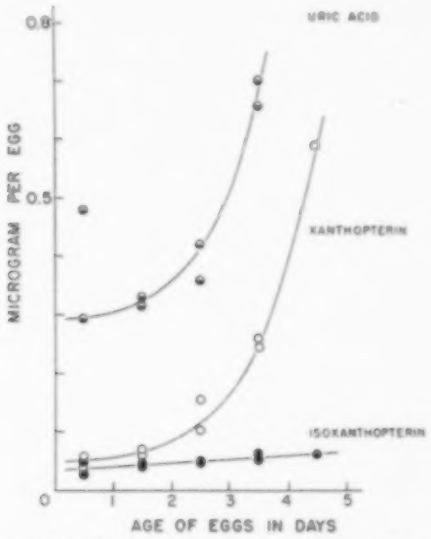


FIG. 4. Changes in pteridine and uric acid content of *Oncopeltus* eggs during embryonic development.

The pteridine content of eggs is given in Fig. 4, and the data for larvae and adults are given in Fig. 5. The xanthopterin content of *Oncopeltus* eggs undergoes a significant increase during embryonic development. This increase is logarithmic in nature. Increases in xanthopterin content are correlated with increases in the content of uric acid, although the correlation is not a 1:1 ratio. Increases in xanthopterin content are also accompanied by increases in the yellow fluorescent material, Zone B (R_f=0·15 in *sec*-butanol:formic acid) and decreases in amounts of Zone G, a fluorescent substance presumed to be lumichrome (BARTEL *et al.*, 1958). *Iso*xanthopterin does not undergo significant increase during development of the egg. The apparent slight increase lies well within the range of experimental error. If the identification of lumichrome is valid, the present results are similar to those reported by BURGESS (1949) for developing eggs of *Melanoplus differentialis* (Thos.). Total pteridine content increases during egg development and this increase is correlated with decreases in content of flavins (lumichrome). Although total pteridine content increases, this increase is due primarily to one compound, xanthopterin, whereas the remaining pteridine, *iso*xanthopterin, shows no significant changes.

The increase of only one pteridine during embryonic development is not continued in postembryonic stages. Increases in amounts of total xanthopterin

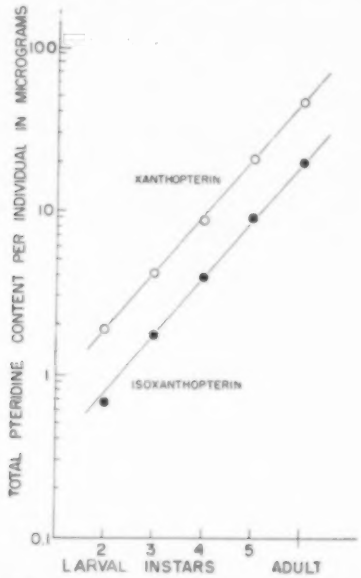


FIG. 5. Changes in pteridine content of *Oncopeltus* individuals during postembryonic development.

and *iso*xanthopterin in individuals during postembryonic development are logarithmic, following Dyar's rule, and ratios of xanthopterin to *iso*xanthopterin remain the same during the larvae and adult instars.

Tissue levels of pteridines

The ratio of xanthopterin to *iso*xanthopterin (Table 3) is approximately $2{\cdot}0 \pm 0{\cdot}3$ for all tissues and body parts of adults, except in the case of fat body and midgut. The unique localization of 2-amino-4-hydroxypteridine in the midgut, the predominance of *iso*xanthopterin over xanthopterin, and the changes occurring in pteridine and flavin levels during starvation and subsequent feeding (Table 4) should be considered in theorizing about synthetic sites for pteridines. It seems reasonable to assume that since pteridines are not detectable in the food materials of *Oncopeltus*, the synthesis of 2-amino-4-hydroxypteridine occurs during some phase of the digestive or absorptive process. *Iso*xanthopterin could then be formed by oxidation of the 2-amino-4-hydroxypteridine as in *Drosophila melanogaster* Meig. (FORREST and MITCHELL, 1955).

Possible synthetic pathways for the formation of xanthopterin or the xanthopterin precursor are not indicated by the data presented. It is quite possible, however, that more work on pteridine metabolism in *Oncopeltus* embryos might help in solving this problem.

TABLE 3—PTERIDINE CONTENT OF TISSUES AND BODY PARTS OF ADULT *Oncopeltus*

Tissue	Micrograms per individual*			Ratio
	*Iso*xantho-pterin	Xantho-pterin	2-amino-4-hydroxy-pteridine	Xanthopterin/ *Iso*xanthopterin
Abdominal hypodermis	9·15	18·2	—	1·99
Fat body	3·08	13·60	—	4·41
Hindgut and Malpighian tubules	0·48	1·08	—	2·25
Midgut and foregut	0·68†	0·16†	0·44†	0·24
Gonads	1·45	2·71	—	1·87
Head	1·74	3·07	—	1·76
Thorax	1·93	3·81	—	2·02
Total	18·51	42·63	—	2·30

* Adult *Oncopeltus* weigh about 0·04 g.
† Varies according to nutritional state of individuals.

VOL. 3 1959

TABLE 4—EFFECT OF STARVATION AND SUBSEQUENT FEEDING ON PTERIDINE CONTENT OF *Oncopeltus* MIDGUT TISSUE

Sample	Pteridine content micrograms/midgut			Additional fluorescent materials R_f 0·55 in *sec*-butanol : formic acid : water (flavin)
	*Iso*xantho-pterin	2-amino-4-hydroxy-pteridine	Xantho-pterin	
(1) a. 1 week starvation	0·03	0·03	0·05	none
b. Control 1 week starvation	0·02	0·04	0·02	none
(2) a. Fed 1 day	0·05	0·33	0·105	+
b. Control (continued starvation)	0·01	0·01	0·02	none
(3) a. Fed 2 days	0·08	0·56	0·10	++
b. Control	0·015	0·035	0·015	none
(4) a. Fed 4 days	0·13	0·82	0·14	++++
b. Control (50% mortality)	0·01	0·01	0·01	none
(5) a. Fed 8 days	0·12	0·49	0·11	+++
b. Control	—	—	—	—

Excretion

Early investigations of pteridine metabolism in insects suggested that certain pteridine-containing insects did not excrete pteridines (BECKER, 1937). Later workers reported the excretion of pteridines by larvae of *Bombyx mori* (ARUGA *et al.*

1951). In the present work, there were no apparent differences in pteridine excretion by larvae and adults of *Oncopeltus*. Results indicate, however, that uric acid is the major nitrogenous excretory product of *Oncopeltus*, accounting for 3–5·5 per cent (dry weight) of total faecal material (Table 5). Pteridines account

TABLE 5—PTERIDINE AND URIC ACID CONTENT OF LARVAL AND ADULT EXCREMENT OF *Oncopeltus*

Source of excrement	Content of excrement mg/g			Ratio pteridine: uric acid
	Xantho-pterin	*Iso*xantho-pterin	Uric acid	
Larvae	1·17	0·82	31·9	0·063
Adults	1·23	0·89	34·9	0·061

for approximately 0·2 per cent of total faecal material. This amount of pteridine, although much less than the amount of uric acid excreted, is very high in comparison with the amounts of pteridine reported in mammalian urine (FLOYSTRUP *et al.*, 1949; RAUEN and HALLER, 1950; CARTER and HORRIGAN, 1953). The xanthopterin is present in a bound form which may be similar to that present in the body. The finding is reminiscent of the labile xanthopterin precursor reported in human urine (KALCKAR, 1949).

SUMMARY

(1) Measurement of total fluorescence of pteridine spots on paper chromatograms permits quantitative estimation with an accuracy of 5 per cent on a 3·0 ml μg sample. Recovery of added pteridine is about 95 per cent.

(2) During embryonic life *iso*xanthopterin does not change, xanthopterin increases 10 fold from 0·05 μg p/egg, and uric acid increases about 3 fold from 0·3 μg p/egg.

(3) During postembryonic development the content of xanthopterin and *iso*xanthopterin increases logarithmically, the ratio remaining constant.

(4) During starvation the amount of 2-amino-4-hydroxypteridine in the midgut is markedly reduced and returns to normal on subsequent feeding. There is a much smaller change in the amounts of xanthopterin and *iso*xanthopterin during starvation.

(5) The fat body contains about 20 per cent of the total *iso*xanthopterin and 30 per cent of the xanthopterin found in the insect. Nearly 50 per cent of the pteridines are in the hypodermis.

(6) 2-amino-4-hydroxypteridine is found only in the midgut, whereas the other pteridines are found in all body tissues.

(7) The levels of *iso*xanthopterin and bound xanthopterin in the faeces of larval and adult *Oncopeltus* are the same. Total pteridines account for about 0·2 per cent and uric acid 3·0–5·5 per cent of the total faecal material.

REFERENCES

ALBERT A. and WOOD H. C. S. (1952) Pteridine syntheses. I. Leucopterin and xanthopterin. *J. appl. Chem.* **2**, 591–592.

ALBERT A. and WOOD H. C. S. (1953) Pteridine syntheses. II. *Iso*xanthopterin. *J. appl. Chem.* **3**, 521.

ARUGA H., YOSHITAKE N., and ISHIKAWA S. (1951) Studies on Xanthopterin-B and Leucopterin-B in the silkworm. *J. seric. Sci., Tokyo* **20**, 399–402.

BARTEL A. H. (1954) A study of uric acid and pterins in certain Hemiptera. Dissertation Ph.D., Univ. California Library, Berkeley, California.

BARTEL A. H., HUDSON B. W., and CRAIG R. (1958) Pteridines in the Milkweed Bug, *Oncopeltus fasciatus* (Dallas). I. Identification and localization. *J. Ins. Physiol.* **2**, 348–354.

BECKER E. (1937) Das Fehlen der Pterine in den Excrementen pterinführender Insekten. *Hoppe-Seyl. Z.* **246**, 177–180.

BURGESS L. E. (1949) A quantitative study of pterin pigment in the developing egg of the grasshopper, *Melanoplus differentialis*. *Arch. Biochem.* **20**, 347–355.

CARTER C. E. and HORRIGAN D. L. (1953) Isolation of a pteridine from urine in leukemia. *Fed. Proc.* **12**, 187.

FLOYSTRUP T., SHOU M. A., and KALCKAR H. M. (1949) Urinary excretion of a pterin after administration of pteroylglutamic acid. *Acta Chem. Scand.* **3**, 985.

FORREST H. S. and MITCHELL H. K. (1955) Pteridines from *Drosophila*. III. Isolation and identification of three more pteridines. *J. Amer. chem. Soc.* **77**, 4865–4869.

KALCKAR H. M., FLOYSTRUP T., and SCHOU M. A. (1949) Enzymic fluorimetric analysis of urinary pteridine derivatives. *Proc. 1st Int. Congr. Biochem., Cambridge*, 75.

MCDONALD H. J. (1955) *Ionography*. Year Book Publishers, Chicago.

RAUEN H. M. and HALLER C. V. (1950) Uber die Ausscheidung von Xanthopterin nach Verabreichung von Pteroylglutaminsäure beim Menchen. *Hoppe-Seyl. Z.* **286**, 96.

YEAGER J. F. and GAHAN J. B. (1937) Effects of the alkaloid nicotine on the rhythmicity of isolated heart preparations from *Periplaneta americana* and *Prodenia eridania*. *J. agric. Res.* **55**, 1–19.

VOL.
3
1959

INTERNATIONAL SYMPOSIUM ON THE PROBLEMS OF ONTOGENETIC DEVELOPMENT OF INSECTS, WITH SPECIAL REFERENCE TO EXPERIMENTAL WORK

On the occasion of the Second Czechoslovak Entomological Conference, arranged under the auspices of the Biological Section of the Czechoslovak Academy of Science and in collaboration with other institutions, an international symposium concerning the problems of ontogenetic development of insects with special reference to experimental work will be held in the first half of September 1959 in Prague.

All applications for attendance and for lectures should be sent to the Secretariat of the Symposium: Entomological Laboratory, Czechoslovak Academy of Science, 7 Vinicna, Prague 2, Czechoslovakia. Invitations with the preliminary programme will be sent in the first quarter of 1959.

Secretariat of the Symposium

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 75 *to* 85. *Pergamon Press Ltd., London. Printed in Great Britain*

PHYSIOLOGY OF DIAPAUSE IN THE ADULT COLORADO BEETLE (*LEPTINOTARSA DECEMLINEATA* SAY)—I THE PHOTOPERIOD AS A CONTROLLING FACTOR

J. DE WILDE, C. S. DUINTJER, and L. MOOK

Laboratory of Entomology, Agricultural University, Wageningen

(*Received* 14 *July* 1958)

Abstract—In a study of diapause in the adult Colorado beetle, the photoperiod has been used as a controlling factor. Details are given of the relation between the photoperiod and the percentage of diapause. Temperature plays a minor role. The sensitive stage is the newly-emerged adult beetle, but treatment in the larval stage has some influence. Some effects on the ovaries are described. Elimination of the eye function does not remove the photoperiodic response. The importance of the feeding rhythm in connexion with the photoperiod is discussed.

VOL. 3 1959

INTRODUCTION

INSECT diapause has been studied most successfully at its onset and termination. Rapid advances have been made by the use of chilling as a diapause-breaking agent. Few attempts have been made to analyse diapause during its establishment. Although many papers deal with the ecology of diapause, factors like the photoperiod have only rarely been applied in physiological studies. By varying this factor one can produce predictable numbers of insects of the desired physiological condition. Different physiological functions can now be correlated during the onset of diapause. "Conditioning" the experimental animals is also of great use in surgical experiments. These considerations have led us to study the causal factors governing the initiation of diapause in the Colorado beetle. In this insect, diapause takes place in the adult stage. This condition is not only characterized by arrest of oögenesis but also by adaptive behaviour changes difficult to define physiologically (DE WILDE, 1954).

In a former paper (DE WILDE, 1954) it was explained why in our opinion the condition of dormancy in the adult Colorado beetle should be called diapause, as proposed by GRISON (1944). Since then DAVEY (1956) has obtained interesting data on a comparable case of dormancy in the sweet clover weevil, *Sitona cylindricollis* Fahr. From these results it also appears that larval and adult diapauses are very much related. For reasons of simplicity, we therefore prefer to maintain the term diapause instead of using special terms like dormancy or "Ruhestadium" (PRECHT, 1954). This has in fact been done by LEES (1955) in his comprehensive review of the literature on insect diapause. In the older literature on the Colorado beetle (TOWER, 1906; BREITENBECHER, 1918; FINK, 1925; KOZLOVSKY, 1937;

SWINGLE, 1941) several possible causes of diapause are mentioned. It is, however, obvious that these authors have overlooked the major effect of the photoperiod. This effect has been reported briefly by us on several occasions (DE WILDE, 1954, 1955, 1956; see also LEES, 1955). It has been found independently by JERMY (1956) and by GORYSCHIN (1956).

Effect of different photoperiods and temperatures

In order to study the combined effect of several photoperiods and temperatures, use was made of three temperature-regulated containers placed parallel to each other, each divided into three light-tight compartments. Three pairs of 40 W

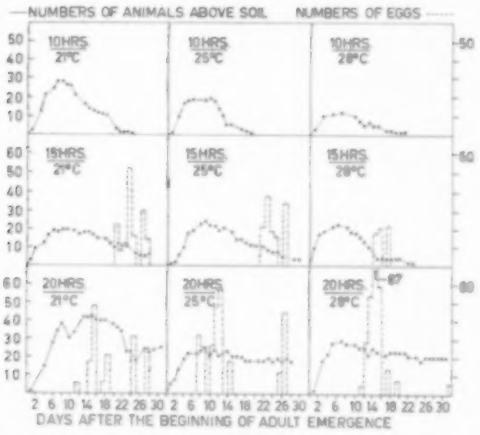

NUMBERS OF ANIMALS ABOVE SOIL
NUMBERS OF EGGS
10 HRS 21°C
10 HRS 25°C
10 HRS 28°C
15 HRS 21°C
15 HRS 25°C
15 HRS 28°C
20 HRS 21°C
20 HRS 25°C
20 HRS 28°C
87
2 6 10 14 18 22 26 30 2 6 10 14 18 22 26 30 2 6 10 14 18 22 26 30
DAYS AFTER THE BEGINNING OF ADULT EMERGENCE

FIG. 1. Diapause and oviposition in beetles reared *ab ovo* under different photoperiods and temperatures. The curves denote the number of beetles present above soil as a function of adult emergence and diapause.

fluorescent tubes were placed crosswise above these compartments and isolated optically. Each pair was controlled by a switch-clock. In this way, the effect of nine combinations of temperature and photoperiod could be studied simultaneously. Illumination did not raise the temperature in each container by more than 1°C. This error was accepted after preliminary experiments with water-cooled incandescent bulbs had shown that temperature had very little influence on the photoperiodic effect.

In a first experiment, temperatures of 21°, 25°, and 28°C were used in combination with photoperiods of 10, 15, and 20 hr. These and the following experiments were made with groups of 20–40 beetles fed with freshly-cut potato sprouts placed in water and changed daily. The intensity of illumination varied between 250 and 1000 Lux. Larvae were reared *ab ovo* under these conditions and allowed to pupate in damp sand at the bottom of the containers. The adult beetles later on burrowed in the same soil to enter diapause. After emergence from the soil, the adults were counted daily as also were the eggs laid. The results are given in Fig. 1.

At 25°C and a 10 hr photoperiod, diapause begins 8–10 days after emergence. During this pre-diapause, marked changes occur in geotactic and phototactic behaviour (DE WILDE, 1954). No oviposition is observed. With photoperiods of 15 and 20 hr this takes place, but only after a pre-oviposition period usually of longer duration than the pre-diapause period. The behaviour of the beetles at a 15 hr photoperiod shows that in individual beetles oviposition may be followed by diapause.

During this experiment (from fifteenth to twenty-fourth day) our supply of young leaves was suddenly cut down by potato blight. It was supplemented by senescent leaves from other sources. This accounts for the gap in oviposition

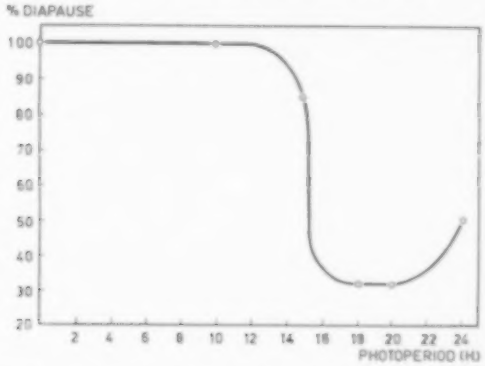


FIG. 2. Percentage of diapause in groups of beetles reared under different photoperiods at 25°C.

shown in the 20 hr group, and is in accord with the findings of GRISON (1952). It probably had some influence on the rate of diapause.

It was of interest to study the effect of temperatures below 21°C. As shown in an earlier paper (DE WILDE, 1950), larvae can hardly be grown at 17°C. We therefore started with third stage larvae previously bred at 25°C. At 17°C, starting with 50–60 larvae, we obtained after 5 weeks 10–15 adults, which were subsequently kept under observation for 40 days. The results clearly demonstrated that oviposition was not suppressed by the low temperature. With a photoperiod of 20 hr at least 50 per cent of the beetles remained active and reproduced.

It follows from these observations that even a supra-optimal temperature (28°C) does not interfere with the "short day" effect. The effect of "long day" is not markedly changed by suboptimal temperatures (17°C). In short, temperature appears to play a minor role. Further experiments with permanent light and darkness and photoperiods of 16 and 18 hr were therefore made at 25°C. The results are shown in Fig. 2. While under permanent illumination, 50 per cent of the beetles enter into diapause. The effect of permanent darkness on diapause behaviour is equal to that of a 10 hr photoperiod. Generally, however, beetles reared *ab ovo* in constant darkness show some oviposition during the prediapause stage.

It may be stressed here that we never succeeded in obtaining generations with 100 per cent active beetles. Always at least 20–30 per cent entered into diapause. On the other hand, photoperiods of 10 hr or less always resulted in 100 per cent diapause. The question thus arises whether this is due to an inherited photoperiod independence of diapause present in 20–30 per cent of the population, or whether the exact optimal conditions for diapause-free development have not been found. We intend to study this point in further experiments.

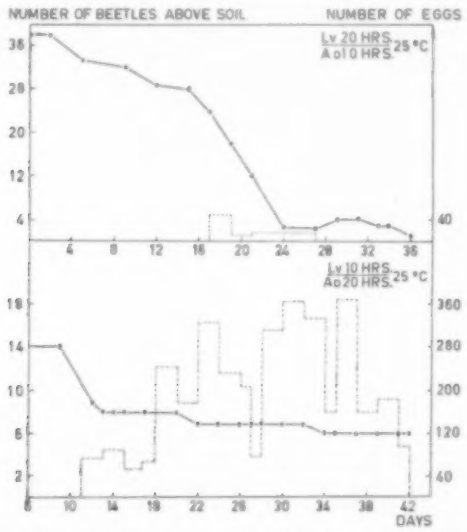


FIG. 3. Diapause and oviposition in groups of beetles treated differently as larvae and adults. The conditions under which the adult is reared are decisive. Only the descending part of the curves is shown.

The sensitive stage

To study the effect of larval and adult treatment on the diapause percentage, larvae were reared at a 10 hr photoperiod, the adults being subsequently kept at 20 hr. Also the inverse was done (Fig. 4). Furthermore, larvae were reared under 10 and 20 hr photoperiods, the adults being kept in constant darkness (Fig. 5). The effect of permanent darkness can be described as a "mild short-day effect" and the behaviour of the adults kept under these conditions is strongly influenced by larval treatment.

As is shown in the diagram, the behaviour of the adult beetles kept under a 10 hr or 20 hr photoperiod is mainly influenced by the treatment of the adult beetle. Reproductive activity is effected by larval treatment only if the female adult is kept in constant darkness. This suggests that the activity of the ovaries in the adult beetle is partly determined by the photoperiod at which the larva is kept. This was further elucidated by studying the excised ovaries of the beetles which had entered into diapause under "short day" conditions, but had been reared as a

larvae under "long day" conditions (20 hr). The ovaries of such beetles invariably contained ova in different stages of vitellinization. In contrast, beetles grown up *ab ovo* under "short day" conditions entered into diapause with a completely inactive vitellarium.

It may be concluded from these observations that there is a cumulative effect of larval and adult treatment on the female reproductive system. As regards

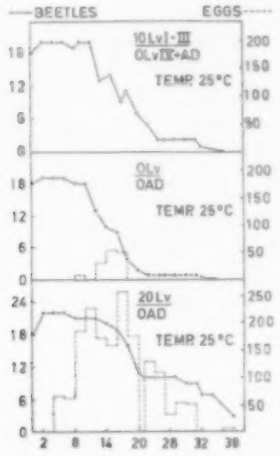


FIG. 4. Influence of constant darkness applied to the adult when larvae are treated differently.

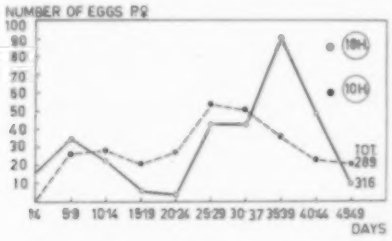


FIG. 5. Rate of oviposition in two groups of post-diapause beetles reared under 10 and 18 hr photoperiods at 25°C, showing that the beetle, immediately after break of diapause, is insensitive to the photoperiod.

diapause behaviour, the treatment of the young adult beetle is decisive. We subsequently studied the photoperiod sensitivity of newly emerged "post-diapause" beetles. Ecologically, this is of importance because in a large part of its geographic area the beetle may end its diapause under "short day" conditions.

Figure 5 shows the result of an experiment in which the oviposition of two groups of 10 post-diapause females was compared, reared respectively under 10 and 18 hr photoperiods. The rate of oviposition was the same, and none of the

beetles re-entered into diapause. Though this proves that the beetle immediately after break of diapause is insensitive to the photoperiod, this should not be extended to the remaining part of adult life. In single cases beetles may hibernate a second time; in rearing experiments in outdoor-cages we have found that this may occur in August as soon as "short day" conditions prevail. It thus remains possible that later in the season the beetles show a renewed photoperiod sensitivity.

Reversibility of the photoperiodic effect

In several experiments we have exposed beetles which had gone into diapause to "long day" illumination. These beetles had been reared under "short day" conditions and were placed daily on soil to observe the beginning of diapause behaviour. Beetles exposed to "long day" immediately after they showed the first burrowing responses but could still feed (DE WILDE, 1953) could in several cases be reversed to reproductive activity. Three or four days later, however, this was no longer possible. Normally beetles going into diapause will hide themselves in soil or other suitable substrates and thus avoid the influence of light. Ovipositing young beetles may at any time be reversed to the diapause condition by applying "short day" illumination. Beetles thus treated at 25°C will stop oviposition and within 3–5 weeks enter into diapause.

Photoperiod and ovarian activity

The ovarioles of *Leptinotarsa* are of the telotrophic type. Primary oöcytes are present in full numbers at the time of the adult moult, and occur throughout the cylindrical germarium. Nutritive cells are inconspicuous, and their cytoplasmic connexions with the oöcytes after being released, though easily detectable in other insects, have not been found by us by the use of the Heidenhain technique nor by Masson's trichrome stain. Clearly the yolk supply of the oöcyte is soon effected by the egg follicle formed by the vitellarium.

Follicle formation only occurs in beetles reared under "long day" conditions, and this is true for both the diapausing and ovipositing fraction. In this case, the vitellarium soon shows a series of 4 or 5 ova in successive stages of completion (Fig. 6). In the diapausing fraction of a "long day" population, and also in beetles subsequently transferred to "short day" conditions, the egg follicle soon disintegrates (Fig. 7). Part of its cells change their function and take part in ovisorption. The yolk soon breaks into globules and gradually disappears leaving an orange-coloured crystalline mass most probably containing part of the β-carotene present in the yolk. In beetles reared under "short day" conditions follicles are rarely found. In some cases we have been able to detect oöcytes in an early stage of yolk supply in the vitellarium of beetles thus treated. This suggests that ovulation may occur under conditions inhibiting follicle formation.

Perception of the photoperiod

The effect of constant darkness was described above as a "weak short day effect". Starting from this observation, we blinded a series of beetles directly

VOL.
3
1959

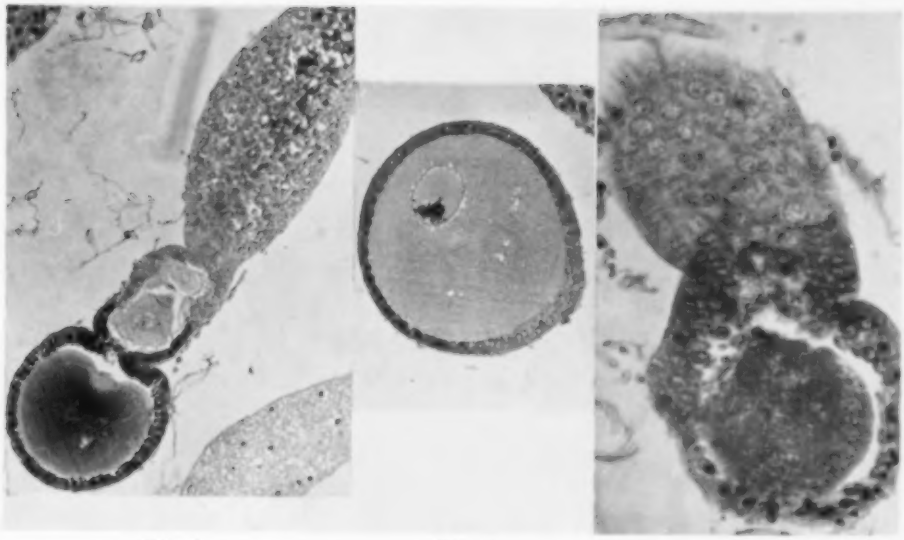


Fig. 6 Fig. 7 Fig. 8

FIG. 6. Section of an ovariole with three primary oöcytes in successive stages of yolk deposition. Heidenhain's hematoxylin.

FIG. 7. Nearly complete egg enveloped in a follicle of well-orientated cubic epithelium. Beetle reared under "long day" conditions.

FIG. 8. Ovisorption by the disintegrating egg follicle from a beetle reared under "short day" conditions. Note the pycnotic nuclei and the irregular shape of the former oöcyte.

after the pupal-adult ecdysis. Blinding was done under CO_2 following two methods: (a) The eyes were cauterized by means of high-frequency alternating current. We used a commercial apparatus (Elter apparaat) for surgical use, with an output of 0–2 W at a frequency of 100 M Herz. A stainless steel microneedle was used as the active electrode. (b) The eyes were covered by black paint made from nailpolish mixed with carbon black. In both cases the result was controlled by following the phototactic behaviour of the beetles when placed in a dark room in a horizontal beam of light. Young beetles are characterized by a strong positive phototaxis.

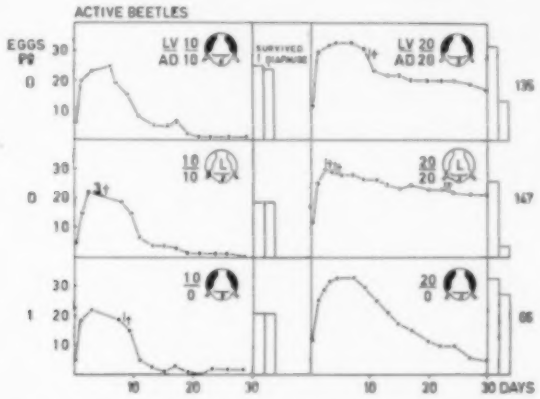


FIG. 9. Result of experiments with blinded (L) and normal beetles, with different larval and adult treatments. The number of beetles above soil from the beginning of adult emergence is shown. The eyes were covered immediately after the beetles emerged from the soil.

Controls were made every two days. "Positive" beetles were excluded from further observation (cauterized series) or repainted ("painted" series).

After careful cauterization, the eye function never recovered but mortality was considerable. This mortality occurred gradually and even went on after 2–3 weeks. We will therefore only mention here the results of the experiments with "painted" beetles. The surviving fraction of the cauterized beetles behaved substantially the same. In Fig. 9 the results are given of an experiment in which two groups of beetles obtained from larvae reared under 10 and 20 hr respectively were treated in different ways: (a) adults reared under 10 and 20 hr photoperiods; (b) adults blinded and subsequently reared under 10 and 20 hr photoperiods; and (c) adults kept in constant darkness.

It is clear that the effect of blinding differs from that of constant darkness. It also appears that blinding has no influence on the photoperiodic effect. Although these experiments demonstrate that the photoperiod can have an effect even when the eyes are excluded, they certainly do not prove that the eyes normally do not take part in the perception of the photoperiod.

Interaction between feeding and photoperiod

An experiment originally designed for studying the site of perception of the photoperiod led us to the importance of feeding for the photoperiodic effect. The principle was to supplement a basic 10 hr photoperiod in a group of beetles by 10 hr illumination of the abdomen only and, in a second group, of head and prothorax only. This was done by fixing the beetles during the supplementary period between the prothorax and the elytra in a black rubber membrane stretched over a blackened glass tube. During this period group A had the abdomen inside and group B outside the tube. Group C was only kept starving during the supplementary 10 hr period in a clear glass tube and served as a control. During a dark period of 4 hr and the following "basic" illumination of 10 hr all three groups of beetles were let free on foliage in an insectary. During "fixation" the beetles could not feed. The experiments were made with 30 newly emerged beetles and lasted 21 days. It was hoped to observe that either group A or B would behave as "short day"-beetles, the other groups as "long day"-beetles. To our surprise the following result was obtained:

TABLE 1

	No. surviving	In diapause	Active
A	7	7	0
B	7	7	0
C	9	8	1

We subsequently made a series of experiments in which groups of 20 beetles (10 ♀♀+10 ♂♂) were subjected to different photoperiods combined with different

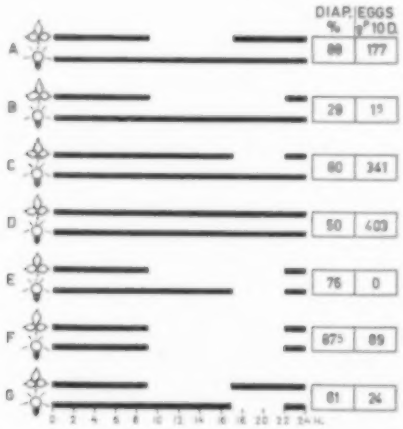


FIG. 10. Result of the experiments with groups of beetles subjected to different photoperiods combined with different "feeding periods".

"feeding periods". During the period of inanition, the beetles were kept in glass boxes on moist filter paper. After this, they were placed on potato sprouts in small insectaries. All experiments were made at 25°C. The results are given in Fig. 10. Although it is difficult to explain the incidence of diapause in each case, it is clear that the effect of the photoperiod, described in the preceding paragraphs, is only shown by beetles with a constant food supply. The experiments also suggest that if light and food coincide for 10 hr per day or less, reproduction diminishes and the percentage of diapause is increased.

DISCUSSION

Like most insects studied, the Colorado beetle belongs to the "long day" type. The curve representing the diapause percentage at different photoperiods has its transition point at 15 hr. It must be stressed here that we have only studied beetles of Dutch origin. A recent study of DANILIEVSKY (1957) shows that the transition point may depend strongly upon the geographic latitude at which an insect naturally occurs.

VOL. 3 1959

As the Colorado beetle has been imported in Europe as recently as 1918, the formation of geographic races may still be in its beginning. In this connexion it may be mentioned that the data obtained by JERMY (1956) with beetles of Hungarian origin are in full accord with ours. Though he only applied photoperiods of 14 and 17 hr, the transition point seems to be in between these values. In the Russian material studied by GORYSCHIN (1956) it is perhaps slightly higher (near 16 hr), but the significance is doubtful. It is remarkable that temperature has little influence on the photoperiodic effect, at least within the range studied. Also in other respects temperature relations of diapause in the Colorado beetle are different from many other insects. We may refer here to the fact that low temperature treatment is ineffective in breaking diapause (DE WILDE, 1954). This is possibly connected with the subtropical origin of the genus *Leptinotarsa*, which has only recently spread to northern regions (TOWER, 1906).

Ovisorption as a consequence of short photoperiods explains the fact that many hibernating females contain ova in different stages of degeneration. Apparently the process of yolk supply is primarily effected. This points to the role of the corpora allata, of which we have also found other evidence (DE WILDE, 1954, 1955). We will treat this point in a following paper.

The importance of food as a controlling factor of diapause in *Leptinotarsa* has been mentioned before by FABER (1949), who states that in newly emerged beetles diapause may be induced by starvation. In addition, we have found that diapause is induced if food is withheld for 10 hr per day, even under permanent light or long-day conditions. The question arises as to whether the photoperiod merely acts by determining the feeding rhythm. We have evidence that this simple explanation does not hold. BONGA (1956) in our laboratory followed the feeding behaviour of individual beetles under photoperiods of 10 and 18 hr with 2 hr intervals during the complete 24 hr cycle. Food intake was determined by measuring the leaf surface eaten. As a rule, food consumption suddenly decreased

after the beginning of the dark period, but after 2–4 hr started again and often surpassed the rate found in daytime. Also, from the data given by GRISON (1950) it is clear that at temperatures of 25°C and above, nocturnal food consumption as a rule exceeds the diurnal values.

In the light of the experiments of HARKER (1956) and the observation of GRISON (1943) it is probable that the photoperiod establishes an internal rhythm of activity. The hypothesis seems attractive that this activity only leads to reproduction if the food contains one or more substances present in young potato leaves. In this connexion we may also refer to the observation of JERMY (1956) who, working with intermediate photoperiods, found an increased percentage of diapause when senescent leaves were eaten.

REFERENCES

BONGA H. (1956) The effect of light on oviposition and diapause in the Colorado beetle. (Report) Wageningen. Unpublished.

BREITENBECHER J. K. (1918) The relation of water to the behaviour of the potato beetle in the desert. *Publ. Carneg. Instn.* **263**, 341–384.

DANILIEVSKY A. C. (1957) Seasonal rhythm and intra-specific differentiation in insects. Comm. Leningrad Univ. **21**, 93–105. In Russian.

DAVEY K. G. (1956) The physiology of dormancy in the sweet clover weevil (*Sitona cylindricollis* (Fahr.)). *Canad. J. Zool.* **34**, 86–98.

FABER W. (1949) Biologische Untersuchungen zur Diapause des Kartoffelkäfers (*Leptinotarsa decemlineata* Say). *Pflanzenschutzberichte* **3**, 65–95.

FINK D. E. (1945) Physiological studies on hibernation in the potato beetle, *Leptinotarsa decemlineata*. *Biol. Bull., Woods Hole* **49**, 301–406.

GORYSCHIN N. I. (1956) Über die photoperiodische-Reaktion des Kolorado-käfers (*Leptinotarsa decemlineata* Say). *C. R. Acad. Sci. U.S.S.R.* **109**, 205–208. In Russian.

GRISON P. (1943) Rythmes d'activité chez *Leptinotarsa decemlineata* Say, et leur importance pour l'étude du phototropisme. *Bull. Soc. Zool.* Fr. **68**.

GRISON P. (1944) Remarques sur des phénomènes de diapause vraie observé au stade imaginal chez le Doryphore. *C.R. Acad. Sci., Paris* **218**, 342–344.

GRISON P. (1950) Influence de la température sur l'activité du Doryphore au stade imaginal. *Proc. 8th Int. Congr. Ent. Stockholm*, 226–234.

GRISON P. (1952) Relations entre l'état physiologique de la plante-hôte et la fécondidé du Doryphore. *Trans. 9th Int. Congr. Ent.* **1**, 331–337.

HARKER J. (1956) Factors controlling the diurnal rhythm of activity of *Periplaneta americana* L. *J. exp. Biol.* **33**, 224–234.

JERMY T. and SÁRINGER G. Y. (1955) Die Rolle der Photoperiode in der Auslösung der Diapause des Kartoffelkäfers (*Leptinotarsa decemlineata* Say) und des amerikanischen weissen Bärenspinners (*Hyphantria cunea* Drury). *Acta agr. Acad. Sci. Hungaricae* **5**, 419–440.

KOZLOVSKY S. (1937) Sur le d'écalage des génerations du *Leptinotarsa decemlineata* en milieu artificiel. *Ann. Epiphyt.* **1**, 99–111.

LEES A. D. (1955) *The Physiology of Diapause in Arthropods*. Cambridge.

PRECHT H. (1953) Über Ruhestadien erwachsener Insekten. In Versuche an Kartoffelkäfern (*Leptinotarsa decemlineata* Say). *Z. vergl. Physiol.* **35**, 326–343.

SWINGLE M. C., GAHAN J. B., and PHILLIPS A. M. (1941) Laboratory rearing of certain leaf-eating insects. *J. econ. Ent.* **34**, 90.

TOWER W. L. (1906) An investigation of evolution in Chrysomelid beetles of the genus *Leptinotarsa*. *Publ. Carneg. Instn.* **48**, x+320 pp.

WILDE J. DE (1949) Het experimenteel beinvloeden van het verloop der generaties bij de Coloradokever (*Leptinotarsa decemlineata* Say). *Bijdr. Dierk.* **28**, 543–549.

WILDE J. DE (1950) Développement embryonnaire et postembryonnaire du Doryphore (*Leptinotarsa decemlineata* Say) en fonction de la température. *Proc. 8th Int. Congr. Ent. Stockholm*, 310–321.

WILDE J. DE (1953) Provisional analysis of the imaginal diapause in an insect (*Leptinotarsa decemlineata* Say). *Acta Pharm. néerl. pharm. Physiol.* **3**, 133.

WILDE J. DE (1954) Aspects of diapause in adult insects, with special regard to the Colorado beetle, *Leptinotarsa decemlineata* Say. *Arch. néerl. Zool.* **11**, 375–385.

WILDE J. DE (1955) The significance of the photoperiod for the occurrence of diapause in the adult *Leptinotarsa decemlineata* Say. *Proc. 1st Int. Photobiol. Congr.*

VOL.
3
1959

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 86 *to* 91. *Pergamon Press Ltd., London. Printed in Great Britain*

UTILIZATION AND EXCRETION OF ORGANIC NITROGEN BY THE SILVERFISH, *CTENOLEPISMA LINEATA*

REUBEN LASKER*

Department of Biological Sciences, Stanford University, Stanford, California

(*Received* 18 *July* 1958)

Abstract—Conversion efficiencies of organic nitrogen to live weight have been determined for the silverfish, *Ctenolepisma lineata*. These efficiencies vary from 0·014 to 0·13 mg N/mg live weight between animals and vary similarly within the same animal depending on the diet. The rate of weight gain by animals feeding on a complete diet ranged from 0·25 to 0·67 mg live weight per week. The silverfish can also live for extended periods on a diet of cellulose alone but requires a source of organic nitrogen for growth and other vital processes. Conservation of nitrogen seems to be accomplished by an inherent low turnover of tissue nitrogen when the animal is metabolizing carbohydrates. *C. lineata* may lose from 8 to 20 per cent of its total body nitrogen before expiring.

INTRODUCTION

IT was shown in an earlier study (LASKER and GIESE, 1956) that the silverfish *Ctenolepisma lineata* can digest and efficiently utilize the carbohydrates (cellulose and starch) in its diet, but no information is as yet available on this insect's ability to make use of the organic nitrogen in its diet. This is of interest since the food of the silverfish (e.g. paper) is often low in organic nitrogenous materials. This paper presents quantitative information on the utilization and excretion of organic nitrogen by *C. lineata*.

EXPERIMENTAL METHODS

The methods for keeping silverfish in the laboratory have been described (LASKER and GIESE, 1956). Silverfish are cannibalistic when on a restricted diet, and for this reason they were kept individually in glass vials with food provided as cellulose alone (Whatman No. 43 filter paper) or as a rolled oat grain which had been soaked previously in a solution containing 0·5 per cent yeast extract and 0·02 per cent liver extract concentrate (dipped oat). Oat grains and cellulose strips were dried in an oven at 70°C before being fed to silverfish. Adult animals used in feeding experiments were selected at random. Each of these animals weighed between 15 and 25 mg live weight. Water content of silverfish is approximately 70 per cent.

The animals, their faeces and the food remaining were usually weighed at two-week intervals. Weighing was done on a semi-micro balance sensitive to 0·01 mg. Faeces were not ingested by silverfish at any time, even when no other food was available. Organic nitrogen was analysed by the micro-Kjeldahl technique using a Kirk distillation apparatus (KIRK, 1936). A weighed sample of each oat grain used as food for silverfish was analysed for total organic nitrogen.

* U.S. Public Health Service Research Fellow. Present address: U.S. Fish and Wildlife Service, Scripps Institution of Oceanography, La Jolla, California.

DIPPED OAT AS A COMPLETE DIET

Gain in weight, egg laying, and successive moulting were chosen as criteria for the selection of dipped oat as an adequate diet. On a diet of dipped oat newly hatched larvae moult, develop pigmented scales, and grow successfully even under bacteria-free conditions (LASKER and GIESE, 1956). The data in Table 1 show that

TABLE 1—INCREASE IN LIVE WEIGHT BY ADULT SILVERFISH FED DIPPED OAT
(All weights are given in milligrams)

No.	Initial weight	Final weight	Gain in weight	Time interval	Rate of gain in weight
				(weeks)	(mg/week)
1	16·24	20·20	3·96	6	0·67
2	21·68	28·11	6·43	14	0·46
3	18·56	25·79	7·28	14	0·52
4	20·14	26·01	5·87	14	0·42
5	16·26	19·22	2·96	12	0·25
6	26·10	31·00	4·90	14	0·35
7	22·28	26·90	4·62	8	0·58
8	21·02	27·09	6·07	14	0·43
	Averages		5·44	12	0·46
	Range				0·25–0·67

in periods between moults adult silverfish also gain weight on this diet. Moulting usually occurred between 8 and 14 weeks. The average gain in weight was 0·46 mg per week or a total of 5·44 mg per average 12 week period. In an experiment comparing two groups of 10 animals each, the control group fed only cellulose, the other dipped oat, only one animal was alive in the control group at the end of 14 weeks, while all but one were alive in the oat-fed group after this period of time. In the cellulose-fed group most of the deaths occurred before eight weeks had elapsed. Thus, under the conditions tested, cellulose alone is an inadequate diet.

CONVERSION EFFICIENCY OF INGESTED NITROGEN TO LIVE WEIGHT

Table 2 presents data on the relation of assimilated organic nitrogen to gain in weight. From the data it appears that an actively feeding silverfish requires about 0·06 mg nitrogen to gain 1 mg of live weight. However, variations exist between different animals and within the same animal, depending upon the diet. For example, one silverfish (no. 6, Table 2) starved for nitrogen, when fed oat, soon converted most of the oat nitrogen into its tissues. Thus an average of only 0·03 mg nitrogen was required to produce each milligram of live weight for several weeks after commencement of feeding. This high efficiency was not maintained and at the end of several more weeks 0·13 mg nitrogen was needed by the same animal to produce each milligram of added live weight. Animal no. 7 (Table 2), also starved for nitrogen, showed a similar change.

The percentage of ingested nitrogen assimilated by the animals is also given in Table 2. The average of these figures for a number of animals is 47 per cent.

TABLE 2—NITROGEN ASSIMILATION AND GAIN IN WEIGHT BY ADULT SILVERFISH FED DIPPED OAT

(All weights are given in milligrams)

No.	Gain in weight	Nitrogen consumed	Nitrogen excreted	Nitrogen assimilated	mg N required for gain of 1 mg live weight	Per cent of consumed N assimilated
1	3·96	0·348	0·224	0·124	0·031	37
2	6·43	0·933	0·530	0·403	0·063	43
3	7·23	1·06	0·572	0·490	0·068	47
4	5·87	1·07	0·658	0·410	0·070	48
5	2·96	0·522	0·185	0·337	0·110	65
6a	6·21	0·388	0·210	0·178	0·030	46
6b	2·62	0·724	0·394	0·330	0·130	46
7a	8·00	0·192	0·077	0·115	0·014	60
7b	2·70	0·453	0·353	0·100	0·037	22
8	6·07	0·685	0·277	0·407	0·067	60

Nos. 6a and 7a indicate animals fed dipped oat after a 12-week diet of cellulose.
Nos. 6b and 7b indicate the same animals after feeding on dipped oat for several weeks.

Two animals which were studied after they fed on cellulose showed interesting variations in this percentage (nos. 6 and 7, Table 2). Animal no. 6 assimilated the same percentage of nitrogen throughout (46 per cent) while no. 7 dropped from 60 per cent assimilation to 22 per cent (average 41 per cent).

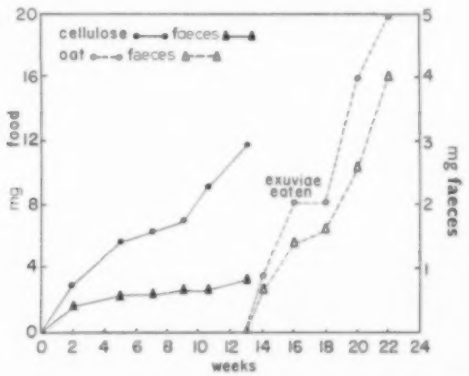


FIG. 1. Food consumption and faecal production of a silverfish.

Several animals fed exclusively on cellulose for 13 weeks were then fed dipped oat. Organic nitrogen intake and excretion was followed over the entire experimental period (22 weeks). Fig. 1 shows the faecal production, cellulose, and oat

intake, and Fig. 2 shows the weight changes of the same animal over the 22 weeks. Although feeding on cellulose was continuous and finally amounted to 12 mg ingested, no sizeable increase in weight was made. Dipped oat was added to the diet toward the latter part of the cellulose feeding period when the animal appeared to be losing weight rapidly. The animal preferentially ate the oat and neglected the cellulose. Within a few weeks a substantial weight gain was made after which

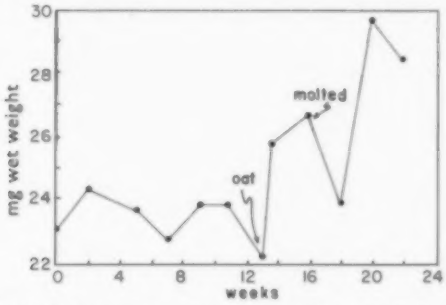


FIG. 2. Weight changes of a silverfish on a diet of cellulose, then oat.

the animal moulted. The exuviae were promptly eaten. During the period of cellulose feeding, faeces production was slight. When oat was eaten, a great increase in faeces (hence excreted nitrogen) was evident. It appears that the silverfish does not excrete nitrogen to any great extent when eating a digestible carbohydrate like cellulose. When fed a rich nitrogen source they excrete more faecal material and more nitrogen, presumably due to more undigested food and increased nitrogen metabolism. The ratio, *weight of nitrogen/weight of faeces*, is about the same with both diets; 0·081–0·129 mg N/mg faeces by eight cellulose-fed animals and 0·079–0·138 mg N/mg faeces by eleven oat-fed animals).

NITROGEN BALANCE OF SILVERFISH ON A COMPLETE DIET

Nitrogen intake and excretion by a number of silverfish was followed for three months. After this time each animal, its faeces and the remaining food were analysed for total nitrogen. In this, as in all of the experiments reported here, it was assumed that nitrogen fixation does not occur in the silverfish. This would seem to be borne out by the generally poorer condition that a silverfish reaches when feeding chiefly on a carbohydrate diet. From the values obtained it was possible to calculate nitrogen intake and excretion for each animal. The data are summarized in Table 3.

In view of the success of ZABINSKI (1929) in rearing cockroaches on a diet of glycine, a similar attempt was made with silverfish. One group of animals was fed dry filter paper previously soaked in 0·1 per cent glycine solution. Analyses were made of nitrogen ingested, excreted and total body nitrogen after the experiment.

The oat-fed group in all cases showed a positive nitrogen balance with an incorporation of nitrogen equal to about half the quantity ingested. Moulting intervened in most cases, and in every instance except those indicated in Table 3

exuviae were eaten by the animal so returning any lost nitrogen to it. It would seem that the exuviae were preferred food because an animal would usually eat them in preference to the dipped oat which was available at the same time. Should the exuviae be lost to the animal, the loss in nitrogen is about 7–8 per cent of the total nitrogen in the animal. LINDSAY (1940) reported a nitrogen loss of 6 per cent for the exuviae of *C. longicaudata*, a related species.

The animals fed cellulose-glycine fared poorly, and at the end of eight weeks all but two of these animals had died.

TABLE 3—NITROGEN BALANCE OF ADULT SILVERFISH FED A COMPLETE DIET
(All weights are given in milligrams)

No.	Initial Animal N	Final Animal N	Ingested N	Excreted N	Lost moult N	Gain in N	Time (weeks)
1	0·462	0·600	0·408	0·224	0·046	0·138	6
2	0·406	0·822	0·946	0·530	—	0·416	14
3	0·863	0·976	0·705	0·592	—	0·113	14
4	0·224	0·714	1·06	0·571	—	0·490	14
5	0·350	0·760	1·07	0·658	—	0·410	14
6	0·233	0·630	0·582	0·185	—	0·397	14
7	0·189	0·909	1·10	0·381	—	0·720	14
8	0·451	0·687	0·520	0·175	0·061	0·284	10

TABLE 4—NITROGEN LOSS BY ADULT SILVERFISH ON A NITROGEN-FREE DIET
(All weights are given in milligrams)

No.	Original live wt	Original Animal N	Final Faeces N	% loss of nitrogen
1	25·62	0·600	0·093	15·5
2	15·67	0·431	0·085	19·8
3	17·51	0·511	0·039	7·6
4	21·20	0·483	0·042	9·5
5	25·38	0·733	0·107	14·6
6	23·51	0·580	0·090	15·5
7	19·58	0·642	0·102	15·7
8	21·05	0·527	0·070	13·1
9	19·34	0·613	0·058	9·4
10	20·15	0·537	0·094	17·3
			Average	13·8

TOTAL NITROGEN LOSS BY SILVERFISH ON A CELLULOSE DIET

Animals fed only cellulose can lose a considerable amount of body nitrogen before expiring. Table 4 gives percentage figures of the total loss of nitrogen by a number of animals fed exclusively on cellulose for several weeks. These animals averaged almost a 14 per cent loss in total body nitrogen before death occurred.

DISCUSSION

The experiments described above show that the usually high carbohydrate diet of the silverfish *C. lineata* must be accompanied by sources of digestible nitrogenous material for growth and other essential metabolic activities. This agrees with the study of LAIBACH (1952) who concluded that *Lepisma saccharina*, another species of silverfish, must have carbohydrate, protein, and fat in its diet for normal growth and development. However, *C. lineata* can survive for a considerable period of time on a diet low in, or devoid of utilizable organic nitrogen. Similarly, COOK and SCOTT (1933) found that termites can exist for considerable periods on pure cellulose. However, for growth they too must have a source of organic nitrogen in the diet.

The low quantity of nitrogen required to produce a gain in live weight indicates a high conversion efficiency by the silverfish, particularly after a prolonged diet of cellulose. The reduced production of faecal material by silverfish on a carbohydrate diet appears to be a combined result of almost complete metabolic utilization of the carbon source (cellulose) and a slow turnover of tissue protein nitrogen; hence an inherent conservation of nitrogen.

The tolerance of silverfish to long periods of nitrogen-lack is not exceptional. Cockroaches can maintain their body weight for many months on a nitrogen-free diet and can be reared on a diet of glycine alone, indicating an elaborate synthetic ability (ZABINSKI, 1929).

The high efficiency with which the silverfish can digest and metabolize cellulose (see digestibility coefficients, LASKER and GIESE, 1956), coupled with its low requirement of organic nitrogen for maintenance and growth, may have adaptive significance. In an environment where cellulose is a predominant substance and where organic nitrogen is limiting, silverfish can compete successfully against forms which are incapable of surviving on a diet of cellulose and low nitrogen. The silverfish has achieved a unique niche by virtue of its ability to utilize cellulose, to survive for long periods of time without organic nitrogen in the diet, and to utilize with high efficiency the organic nitrogen which is available to it.

Acknowledgement—The author is indebted to DR. ARTHUR C. GIESE for his suggestions, advice and help during this study.

REFERENCES

COOK S. F. and SCOTT K. G. (1933) The nutritional requirements of *Zootermopsis* (*Termopsis*) *angusticollis*. *J. cell. comp. Physiol.* **4**, 95–110.

KIRK P. L. (1936) A one-piece glass micro-Kjeldahl distillation apparatus. *Industr. Engng. Chem. (Anal.)* **8**, 223–224.

LAIBACH E. (1952) *Lepisma saccharina* L., das Silberfischchen. *Z. hyg. Zool.* **40**, 1–50.

LASKER R. and GIESE A. C. (1956) Cellulose digestion by the silverfish *Ctenolepisma lineata*. *J. exp. Biol.* **33**, 542–553.

LINDSAY E. (1940) The biology of the silverfish, *Ctenolepisma longicaudata* Esch. with special reference to its feeding habits. *Proc. roy. Soc. Vict.* **52**, 35–83.

ZABINSKI J. (1929) The growth of blackbeetles and of cockroaches on artificial and on incomplete diets. Part I. *J. exp. Biol.* **6**, 360–386.

VOL. 3 1959

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 92 *to* 117. *Pergamon Press Ltd., London. Printed in Great Britain*

THE ROLE OF PHOTOPERIOD AND TEMPERATURE IN THE DETERMINATION OF PARTHENOGENETIC AND SEXUAL FORMS IN THE APHID *MEGOURA VICIAE* BUCKTON—I THE INFLUENCE OF THESE FACTORS ON APTEROUS VIRGINOPARAE AND THEIR PROGENY

A. D. LEES

Agricultural Research Council, Unit of Insect Physiology, Cambridge

(*Received* 19 *July* 1958)

Abstract—Apterous virginoparae from long-established parthenogenetic clones of *Megoura* are capable of producing three types of daughters (winged and wingless virginoparae and sexual oviparae) in addition to males. This polymorphism is regulated by the environment which, by acting through a series of maternal "switch" mechanisms, directs the growing oöcytes and embryos into different developmental pathways.

Although there is little reason to doubt that sex is determined genetically (presumably at the oöcyte stage), the sex ratio of the progeny is also under physiological control. In certain environments, notably at high or low temperatures (although not in long photoperiods), male production is inhibited. But even under the most favourable conditions males are relatively scarce and are born predominantly in the middle of the reproductive life of the parent.

The later differentiation of part-grown female embryos is also governed environmentally. Long photoperiods and high temperatures induce them to become virginoparae, the reverse conditions, oviparae. The action of photoperiods of intermediate or "critical" length is particularly instructive as providing evidence of the maternal, and perhaps humoral, control of these forms. Their differentiation begins when the parent is still in the early larval instars and is completed in the adult stage. The progression of determination can be explained in terms of the competence of embryos in different stages of growth to respond to alternative maternal stimuli.

INTRODUCTION

It has long been known that in many aphids the appearance in autumn of the sexual forms is determined by the environment, length of day and temperature being of particular significance. This was first appreciated by Marcovitch (1924) whose work on *Aphis forbesi* was later extended to other aphids without host alternation ("monoecy") such as *Aphis chloris* (Wilson, 1938), *Brevicoryne brassicae* (Bonnemaison, 1951) and *Acrythosiphon pisum* (Kenten, 1955), as well as to several species with host alternation ("heteroecy") like *Aphis fabae* (Davidson, 1929; de Fluiter, 1950) and *Myzus persicae* (Bonnemaison, 1951). The extensive researches of Shull (1928, 1929) on the heteroecious species *Macrosiphum solanifolii* (= *M. euphorbiae*) have not been fully integrated with subsequent work, perhaps because he laid especial emphasis on the role of these agencies in "wing

determination". Yet he, also, made it clear that the production of these same winged forms was a prelude to the appearance of the sexual females in the succeeding generation (SHULL, 1930).

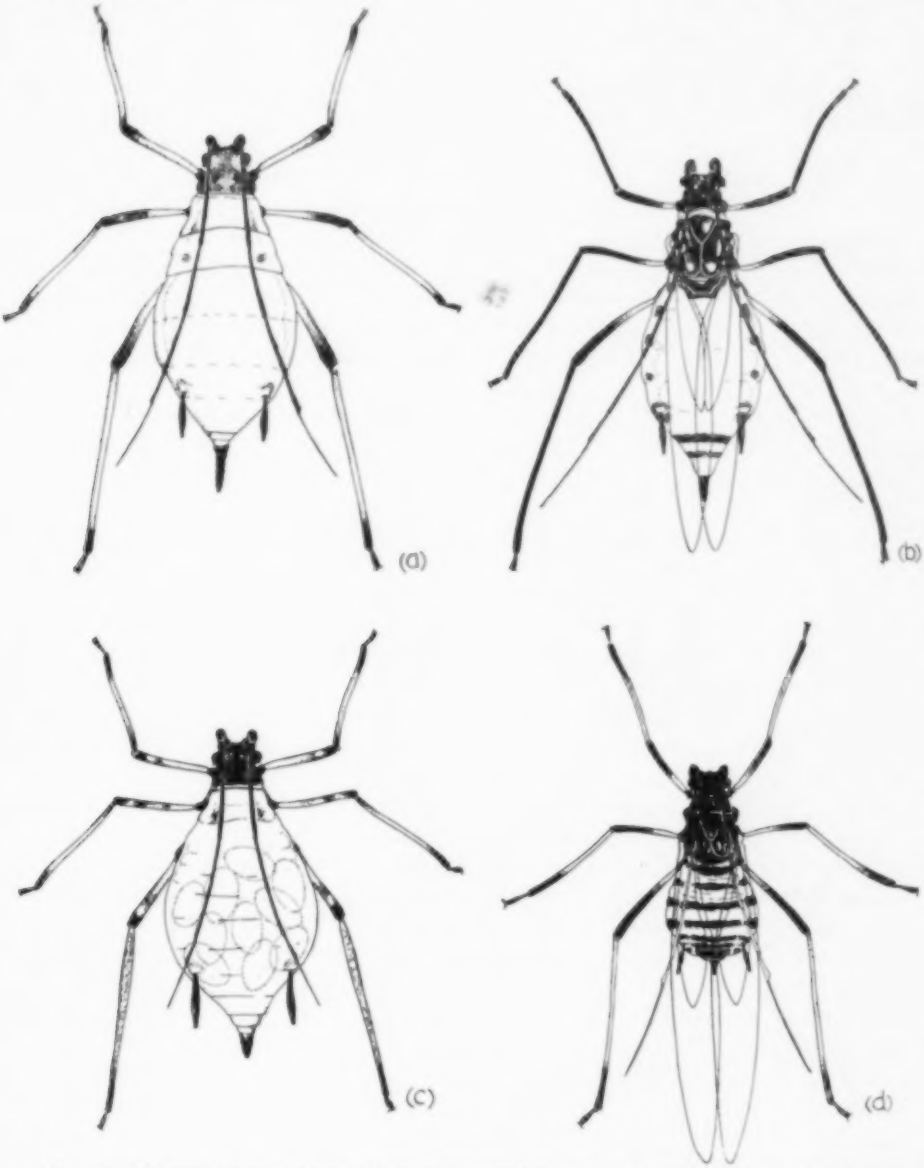


FIG. 1. *Megoura viciae* Buckton. (a) apterous virginopara; (b) alate virginopara; (c) ovipara; (d) male.

VOL. 3 1959

The present work on the physiology of aphid form determination has been undertaken with the object of gaining further insight into the mode of action of the environment. With this end in view, particular attention has been paid to the provision of accurately controlled conditions. The examination of the reproductive capacities of individual aphids has also been an essential feature of this study, for reasons which appear below.

The vetch aphid *Megoura viciae* is monoecious, the normal hosts being perennial legumes of the genera *Lathyrus* and *Vicia*. The forms mentioned in the present context are illustrated in Fig. 1. They are (a) the *apterous virginoparae*—wingless viviparous parthenogenetic females; (b) the *alate virginoparae*—winged parthenogenetic females; (c) the *oviparae*—apterous egg-laying females and (d) the winged *males*. The two latter are the sexual forms. The fifth distinct form, the *fundatrix*, and her immediate descendents (the so-called fundatrigeniae) do not enter into the present enquiry. Here we consider the types of offspring produced when wingless virginoparous parents from a long-established parthenogenetic stock are acted upon by varying conditions of photoperiod, temperature, and nutrition.

METHODS AND MATERIAL

The aphids were grown on germinating broad beans (*Vicia faba*) under lamp glasses, as described by KENTEN (1955). Two points are noteworthy on the technique of rearing. Since preliminary experiments indicated that the control of form is exercised through the maternal physiology, much information would have been lost if the parent virginoparae had been reared as a group. It would then have been impossible to separate their respective "families". This difficulty was therefore avoided by isolating each parent during the reproductive period. It follows also that the usual practice of expressing the incidence of each form in terms of the pooled offspring of many parents is inadequate. What is required is a knowledge of the proportion of *parents* that respond in a particular way. In the present paper the performance of the parent aphids has generally been classified according to whether they gave birth to virginoparae only, oviparae only, or whether they yielded mixed families of virginoparae and oviparae. The reasons for adopting these categories will appear in due course.

Megoura very readily produces alate virginoparae under "crowded" conditions. Since this response is highly variable, there is always some danger of making spurious correlations with other factors unless the production of this form can be suppressed. This was accomplished (except under certain low temperature conditions) by limiting the numbers of larvae developing on a single plant to about 20.

The rearing method was therefore as follows. Two or three young apterous virginoparae from the stock culture were placed on a germinating bean and were allowed to deposit 20 or so offspring overnight under the conditions of the experiment. These individuals (the grandparents) were then removed. After the 20 larvae had become adult they were transferred to separate plants. In order to study the reproductive sequence, each of these parents was moved to a fresh bean plant after about 20 offspring had been born. Since, under these favourable

circumstances, a *Megoura* aptera gives birth to about 100 young, five successive batches of offspring could usually be collected. The classification of the progeny was often carried out at the third instar when the type of reproductive system and the presence or absence of wing buds suffices to distinguish the forms.

The experiments were carried out in a series of plant growth cabinets equipped for controlling temperature and photoperiod. Temperatures constant to $\pm$ 0·5°C were usually employed. Fluorescent "daylight" lamps, operated by time switches, gave a light intensity of 50–100 ft-candles at plant level. The stock of *Megoura* was derived from a colony established by LICKERISH (1953) for use in insecticidal studies. Material was kindly supplied by the Department of Insecticides and Fungicides, Rothamsted Experimental Station, where a duplicate culture has been kept for a number of years in a constant parthenogenetic condition. The line used in the present experiments originated with a single apterous virginopara in 1954 and therefore constitutes a clone. Stock cultures were maintained at 15°C and received a daily light period of 16 hr.

VOL. 3 1959

RESULTS

The photoperiodic induction of oviparae and virginoparae at 15°C

When first instar larvae from the stock culture were reared in typical "long" and "short" photoperiods of 16 and 12 hr respectively, the form of these individuals was not affected in any way: all became apterous virginoparae. But when these adults were subsequently permitted to reproduce under the same environmental conditions, photoperiod was seen to have exerted a decisive influence upon the form of the progeny. Some typical results are given in Table I.

Parents which developed under long day conditions all produced families consisting exclusively of wingless virginoparous females and males. In the short day the parents yielded families consisting exclusively of oviparae and males. It was therefore clear from the outset that the mechanisms of female and male determination must be entirely different, for whereas the type of female shows a complete and clear-cut dependency on photoperiod, approximately the same numbers of males are formed in long and in short days. The question of male determination is discussed at greater length elsewhere (p. 108).

Photoperiods of critical length. It is of considerable interest to discover how parents respond to conditions that are photoperiodically intermediate. Systematic tests showed that a daily photoperiod of 14½ hr was critical for this clone of *Megoura*. Some specimen progeny records are given in Table 2.

The eleven parents reared in this photoperiod all gave birth to both virginoparae and oviparae. Occasionally, as in parent "1", both types of offspring were represented in every batch. Nevertheless, this was rather unusual. As a rule the parent aphids tended to produce only one kind of female offspring at a time. They then "switched over" in an apparently abrupt fashion to the production of the alternative form. This transition occurs at different times in the sequence of offspring. For example, aphid "2" produced only two virginoparae (perhaps among the first to be born) followed by 105 oviparae. The switch has occurred

later in parents "3" to "6". Indeed with parent "6" it was delayed until the end of embryogenesis, since there was a sequence of 65 virginoparae followed by only 6 oviparae. All the parents in this series began their reproductive life as virgino-para-producers.

Permanent darkness was also found to constitute an "intermediate" stimulus. A somewhat longer series of 22 parents was reared, including the typical examples

TABLE 1—PROGENY RECORDS OF INDIVIDUAL APTEROUS VIRGINOPARAE REARED THROUGHOUT DEVELOPMENT AND ADULT LIFE AT 15°C IN LONG (16 HR) OR SHORT (12 HR) PHOTOPERIODS

16 HR PHOTOPERIOD

No. of parent	1			2			3			4			5			6			7		
Batch	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂
1	23			26			25			13			20			18			18		
2	14			17			18			26		2	22			13			20		1
3	1		8	3		2	11			7		2	20		5	16		11	3		3
4	22		5	11		10	14		5	18		5	15			20		1	19		11
5	15			21		1	21		1	16			18			16			25		1
Totals	75		13	78		13	89		6	80		9	95		5	83		12	85		16
Grand totals	88			91			95			89			100			95			101		

12 HR PHOTOPERIOD

No. of parent	1			2			3			4			5			6			7		
Batch	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂
1		17			18			27			26			25			22			18	2
2		19	1		16	2		6	5		10			17			6	3		8	6
3		16	6		4	9		28	12		14	11		6	11		24	11		15	6
4		20	1		14	2		15			17	4		18	2		23			15	
5		24			18			17			2			19			19			7	
Totals		96	8		70	13		93	17		69	15		85	13		94	14		63	14
Grand totals	104			83			110			84			98			108			77		

V. ap., virginoparae apterae; *Ov.*, oviparae; ♂, males.

TABLE 2—REPRESENTATIVE PROGENY RECORDS OF INDIVIDUAL APTEROUS VIRGINOPARAE REARED FROM BIRTH AND DURING ADULT LIFE AT THE CRITICAL PHOTOPERIOD OF 14½ HR

No. of parent	1			2			3			4			5			6		
Batch	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂
1	14	1		2	30		14	8		16			15			15		
2	5	28	2		25	4		32	4	35		1	30			25		
3	9	2	9		19	11		36	1	23		17	18		7	18		13
4	1	10	2		18			14			22		12	10		7	2	1
5		1			13			5			6		1	4			4	
Totals	29	42	13	2	105	15	14	95	5	74	28	18	76	14	7	65	6	14
Grand totals	84			122			114			120			97			85		

V. ap., virginoparae apterae; *Ov.*, oviparae; ♂, males.

given in Table 3. The response varied considerably. Some aphids (e.g. "1" and "2") yielded virginoparae only (apart from the usual quota of males). Some produced only oviparous offspring (e.g. "3" and "4"). The majority, however, again gave birth to females of both types (e.g. "5" to "14"). An examination of their progeny records shows that the sequence may begin with virginoparae and end with oviparae, the change-over occurring at different times in relation to the order

TABLE 3—REPRESENTATIVE PROGENY RECORDS OF INDIVIDUAL APTEROUS VIRGINOPARAE REARED FROM BIRTH AND DURING ADULT LIFE IN PERMANENT DARKNESS AT 15°C

No. of parent	1			2			3			4			5			6			7		
Batch	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂
1	27			15				26			20		4	10		22			13		
2	17		1	18				11	3		13	3		6		18			20		
3	14			20		2		19	3		18	3		12	11	5	2	2	25		2
4	18			20				21			19	1		20	2		5	8	8	5	
5	20		5	3				18	1		8			8			18	12		9	
Totals	96		6	76		2		95	7		78	7	4	56	13	45	25	22	66	14	2
Grand totals	102			78			102			85			73			92			82		

No. of parent	8			9			10			11			12			13			14		
Batch	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂
1		24			12			12		2	14		8	8			25		3	13	
2		22	2		14			14			24			22	1		18			20	2
3	15		10		23	3		23	3	19	1	7	22	1	6	24		6	10	3	6
4	22			9	6		9	6					19			1	15		17		4
5	17			4			4						4				12		7	15	
Totals	54	46	12	13	55	3	13	55	3	21	39	7	53	31	7	25	70	6	37	51	12
Grand totals	112			71			71			67			91			101			100		

V. ap., virginoparae apterae; *Ov.*, oviparae; ♂, males.

of parturition ("5" to "7"). Or the sequence may begin with oviparae and change over later to virginoparae ("8" to "10"). Three parents out of twenty-two gave evidence of a second "switch over" by reverting to the type of offspring produced at the beginning of parturition ("11" to "13"). One parent ("14") even showed three such reversals of function, producing successive groups of virginoparae, oviparae, virginoparae, and finally oviparae again.

These results provide a clear indication of the instability of the physiological system controlling form determination in these intermediate régimes. Further, they suggest that this system is maternal, otherwise one would expect the two types of female to be distributed randomly in the progeny sequence. That the distribution is manifestly non-random can be appreciated even with parents that yield both types, since their progeny are born in groups which are in themselves homogeneous. The separation of the parental generation into virginopara-producers, ovipara-

producers, and producers of both these forms therefore seems the most informative way of classifying the observations.

In Fig. 2 is shown the incidence of these three categories of parents when the latter were reared throughout life in daily photoperiods ranging from permanent darkness to permanent illumination. The curves are based on records of about 200 parents and their 20,000 daughters. It will be seen that the proportion of ovipara-producers remains at 100 per cent in photoperiods ranging from 4 to 12 hr

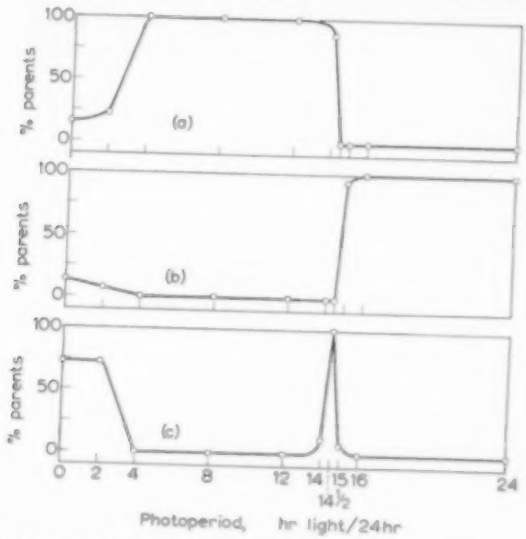


Fig. 2. The influence of photoperiod on the production of virginoparae and oviparae at 15°C. The three graphs show the proportion of parents, in percent, that yielded oviparae only (a), virginoparae only (b), and mixed families of both forms(c).

(Fig. 2a). Even at 14 hr, 88 per cent of the parents yielded oviparae only. With longer photoperiods the percentage falls off very sharply, reaching zero at 15 hr. The same result is observed in all longer days and under conditions of continuous illumination. The proportion of ovipara-producers again falls, though less abruptly, between 4 and 2 hr of light. There appears to be little further intensification of this effect when light is completely absent.

In general, the curve for virginopara-producers is, of course, the inverse of Fig. 2a. Long photoperiods strongly favour the production of virginoparae and short daylengths entirely suppress their appearance (Fig. 2b). The incidence of parents of mixed families is greatest around the critical photoperiod of 14½ hr, although significant numbers also occur if the daily period of light falls below 2 hr (Fig. 2c).

The effect of temperature on the production of oviparae and virginoparae

The interaction of photoperiod and temperature has also been followed by exposing apterous virginoparae during development and parturition to different

constant temperatures in typical "long" and "short" days. The results are summarized in Table 4. Despite the precautions mentioned previously (p. 94), some parents gave birth to a proportion of alatae at low temperatures. A fourth category of parent has therefore been added to those previously mentioned, namely those yielding both winged and wingless virginoparous offspring. Nevertheless, as far as the origin of sexual and parthenogenetic females is concerned, this class is virtually equivalent to the category of parents producing apterae only, since the presence or absence of wings is decided much later in embryogenesis than is the parthenogenetic or oviparous condition (LEES, unpublished).

TABLE 4—THE INFLUENCE OF TEMPERATURE AND PHOTOPERIOD ON PARENTAL FECUNDITY AND ON THE CLASSES OF FEMALE OFFSPRING PRODUCED

Temperature °C	Photoperiod											
	16 hr						12 hr					
	Total parents	Offspring per parent	*V. ap.*	*V. ap.* + *V. al.*	*Ov.*	*V. ap.* + *Ov.*	Total parents	Offspring per parent	*V. ap.*	*V. ap.* + *V. al.*	*Ov.*	*V. ap.* + *Ov.*
25	9	65	100	0	0	0	9	36	100	0	0	0
20	29	110	100	0	0	0	27	102	4	0	29	67
15	25	95	100	0	0	0	37	99	0	0	100	0
11	32	57	66	34	0	0	19	56	0	0	100	0
8*	19	6	68†	0	0	0	13	3	0	0	62†	0

V. ap., percentage of parents producing apterous virginoparae only; *V. ap.* + *V. al.*, percentage of parents producing both apterous and alate virginoparae; *Ov.*, percentage of parents producing oviparae only; *V. ap.* + *Ov.*, percentage of parents producing both apterous virginoparae and oviparae. The mean numbers of offspring include males.

* After final moult parent placed at 20°C.

† Remaining parents produced no offspring.

Table 4 also shows that temperature markedly affects the fecundity of the parents. 25°C is near the upper limit for continuous reproduction. The aphids develop almost normally but gradually darken after the final moult. The progeny is greatly reduced in number, and dissection of the parent reveals large numbers of degenerating embryos. The capacity of the offspring to assimilate the available food is also impaired to a striking extent. Even when the larvae are reared on food at favourable temperatures (e.g. 20° or 15°C) they grow at less than half the normal rate and develop into minute adults often weighing no more than one tenth of the normal weight. Similar effects of heat injury have been described in other aphids (e.g. KENTEN, 1955).

Embryo production is maximal at 15–20°C but is again depressed at lower temperatures. At 8°C slow larval development takes place but no offspring are born, despite the fact that the moribund insects often contain 20 or 30 large embryos. Since it was desired to study the effect of the lowest possible temperature on the form of the offspring, the parents were moved to 20°C after the final moult. This resulted in an average yield of four daughters per parent (Table 4). It should be

noted that the determination of these individuals as oviparae or virginoparae depends on events taking place during the early larval instars, that is, long before the transfer to the higher temperature was made (see p. 106).

The effect of temperature on the form of the offspring may now be considered in detail:

(a) At 25°C. Only one type of aphid, the apterous virginopara, was formed, whether the photoperiod was long or short. Evidently this high temperature completely suppresses the tendency for a short day to favour ovipara-production. Males were always absent.

TABLE 5—PROGENY RECORDS OF INDIVIDUAL APTEROUS VIRGINOPARAE REARED THROUGHOUT LARVAL AND ADULT LIFE AT 20°C IN LONG (16 HR) OR SHORT (12 AND 14 HR) PHOTOPERIODS

16 HR PHOTOPERIOD

No. of parent	1			2			3			4			5			6			7		
Batch	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂
1	25			20			22			30			19			14			29		
2	23			17		2	23		2	19		3	26		1	33			17		2
3	28		2	20		13	28		1	40		6	21		2	26		1	22		5
4	19		3	10			28		2	26		1	21		3	31		2	25		2
5	4			7			15			13			13			12			13		
Totals	99		5	74		15	116		5	128		10	100		6	116		3	106		9
Grand totals	104			89			121			138			106			119			115		

12 HR PHOTOPERIOD

No. of parent	1			2			3			4			5			6			7		
Batch	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂
1		18		27				17		7	21		27			3	20		27	1	
2		8	6	20		1		18	5		17		18		2		20	10	6	11	
3		9	10	11				20	10		17		19			1	13	2	17		1
4		24		21		14	15	1			15	14	17	2	10	21			7	19	16
5		20		21			20				20			35		20				18	
Totals		79	16	100		15	35	56	15	7	90	14	81	37	12	45	53	12	57	49	17
Grand totals	95			115			106			111			130			110			123		

14 HR PHOTOPERIOD

No. of parent	1			2			3			4			5			6			7		
Batch	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂
1	27			27			14			17	5		6	11		3	18		18	8	
2	19		2	1	18	4	19	1	10		19	6		17	11		31	11		22	9
3	12		9		12	8	18		1	9	11	2	16	7	1	17	3		2	15	
4	25		4		30	5	19			29			21			24			18		
5	8				18		11			20			15			12	5		8	10	
Totals	91		15	28	78	17	81	1	11	75	35	8	58	35	12	56	57	11	46	55	9
Grand totals	106			123			93			118			105			124			110		

V. ap., virginoparae apterae; *Ov.*, oviparae; ♂, males.

(b) At 20°C. As at 25°C and 15°C, only apterous virginoparae were born if the parents were reared in long photoperiods (Table 5). Under short-day conditions a temperature of 20°C was intermediate in its action, only partially counteracting the short-day effect. The results were much the same as when the parents were exposed to critical photoperiods at 15°C (p. 95). For example, some families of parents reared in a 12 hr photoperiod consisted of oviparae only ("1", Table 5), others of virginoparae only ("2"). In addition, the same kinds of regularities were noted in the reproductive sequences of those parents (67 per cent of the total) which had mixed female progenies. Thus the parent may begin parturition with a series of oviparae ("3") or a series of virginoparae ("4", "5") before turning over to the production of the alternative form. Other parents show indications of a second reversal ("6", "7"). Some particularly clear instances of double reversals were secured by using a 14 hr photoperiod, that is, one which is just short of the critical length. Nos. "3"–"5" (Table 5) are instances of this kind while parents "6" and "7" provide evidence of three reversals. Although the "inertia" in the maternal control mechanism usually results in the formation of large uniform groups of females, it is worth noting that the numbers in a group may be quite small. Indeed, parent "3" formed only a single ovipara in a sequence of 81 virginoparae. At this temperature males were fairly plentiful.

(c) At 11°C. With respect to the determination of parthenogenetic and oviparous females the results were similar to those obtained at 15°C. The short-day parents yielded oviparae only and the long-day parents virginoparae only (Table 6).

TABLE 6—PROGENY RECORDS OF INDIVIDUAL APTEROUS VIRGINOPARAE REARED THROUGHOUT LARVAL AND ADULT LIFE AT 11°C IN LONG OR SHORT PHOTOPERIODS

16 HR PHOTOPERIOD

No. of parent	1				2				3				4				5			
Batch	*V. ap.*	*V. al.*	*Ov.*	♂	*V. ap.*	*V. al.*	*Ov.*	♂	*V. ap.*	*V. al.*	*Ov.*	♂	*V. ap.*	*V. al.*	*Ov.*	♂	*V. ap.*	*V. al.*	*Ov.*	♂
1	20				25				25				10				12	4		
2	20			3	23			2	24			2	14				17	6		1
3	21			1	25				15				23	4			1			1
Totals	61			4	73			2	64			2	47	4			30	10		2
Grand totals	65				75				66				51				42			

12 HR PHOTOPERIOD

No. of parent	1				2				3				4				5			
Batch	*V. ap.*	*V. al.*	*Ov.*	♂	*V. ap.*	*V. al.*	*Ov.*	♂	*V. ap.*	*V. al.*	*Ov.*	♂	*V. ap.*	*V. al.*	*Ov.*	♂	*V. ap.*	*V. al.*	*Ov.*	♂
1			13				25				23				25				24	
2			11	3			26	5			24	3			14	1			31	1
3			14				12				15				9				20	
Totals			38	3			63	5			62	3			48	1			75	1
Grand totals	41				68				65				49				76			

Note—*V. ap.*, virginoparae apterae; *V. al.*, virginoparae alatae; *Ov.*, oviparae; ♂, males.

But it will be noted that there is also an effect on the type of virginopara. Although 66 per cent of the parents exposed to the 16 hr day produced apterous virginoparae only, 34 per cent had some winged virginoparae among their offspring (Table 4). The progeny records of two such individuals are given in Table 6, "4" and "5".

The aphids were somewhat sluggish at this temperature and were reluctant to cling on after transference to the new plant. As it is known that *Megoura* adults readily yield alatae if isolated from the plant for several hours (LEES, unpublished observations), particular care was taken to ensure that the disturbance to feeding and parturition was as slight as possible. Since some alatae were still formed in spite of these precautions, it appears that low temperature has a definite promotive effect upon wing production. A similar conclusion was reached by KENTEN (1955) in her work on *Acyrthosiphon*. It will be noted that no *Megoura* alatae were produced under short-day conditions (Table 4). This is a result of the comparatively early embryonic determination of the oviparae; such presumptive oviparae are immune to influences that would lead to the production of winged forms by a parent yielding virginoparae (unpublished results).

(d) At 8°C. 19 parents exposed to a 16 hr photoperiod during larval development and then transferred to 20°C after the final moult gave birth to an average of six young, all of them virginoparae (Table 4). Although it is to be expected that a low temperature would have the reverse effect to a high one, and would therefore tend to inhibit the production of virginoparae, this particular low temperature is evidently inadequate to overcome the dominant action of the long day.

Although some effects of low temperature on wing determination were detected at 11°C, no alatae appeared at 8°C. This may have been due to the method of transferring the teneral adults to a temperature of 20°C, since the period of wing determination coincides with the adult life of the parent (unpublished results).

The chronology of embryonic development

Before attempting to relate the period of embryonic sensitivity to photoperiodic stimuli it is necessary to outline the course of embryo production in the growing parent. This information is given in Fig. 3 and Table 7. The stages of embryonic differentiation have not been followed in any detail. But since the embryos develop sequentially from the apical germarium, growth stages can be roughly characterized by the position of the embryo in the ovariole. This, of course, no longer applies when ovulation ceases. Only embryos lying freely in the ovariole were counted: any still attached to the germarium were usually in the oöcyte stage and were therefore disregarded.

Table 7 shows that ovulation begins before birth since the ovarioles of recently born first instar larvae already contain an average of two embryos each. By the third instar four embryos are present in each ovariole and six in the teneral adult (Fig. 3, a–c). After the final moult the aphid feeds for three days at 15°C before parturition begins. During this interval the embryos grow considerably in volume, although the number per ovariole usually remains at about 6 (compare (c) and (d) Fig. 3). If the number of ovarioles is taken as 18 (see below) the total number of

embryos differentiated by the time of the final moult would be 18×6 or 108. Since the mean number of offspring at 15°C was only 97 (Table 4), it would appear that ovulation ceases after the final moult. This may not, however, be an entirely

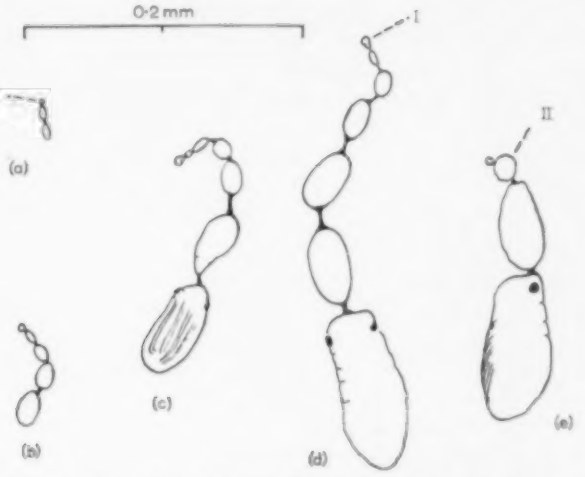


FIG. 3. Outline drawings of typical ovarioles at successive stages in the development of the parent. (a) 1st instar larva; (b) third instar larva; (c) teneral adult; (d) adult at inception of parturition; (e) old adult which has already given birth to 82 young. (I) germarium; (II) degenerating embryo.

correct interpretation, for the examination of virginoparae that have already deposited a proportion of their progeny indicated that more embryos were present than could be brought to maturity. Thus a virginopara which had already given

TABLE 7—OVARIOLE COUNTS IN APTEROUS VIRGINOPARAE AND THE NUMBERS OF EMBRYOS CONTAINED IN EACH OVARIOLE AT DIFFERENT STAGES OF EMBRYOGENESIS

Stage	Numbers of ovarioles			Numbers of embryos	
	No. of ♀♀ examined	Modal no. per ♀	Range	Modal no. per ovariole	Range
1st instar larva	14	18	16–19	2	1–3
3rd instar larva	13	18	16–21	4	3–5
Teneral adult	14	—	—	6	4–7
Adult, parturition beginning	3	—	—	6	5–7

birth to 82 young was found to contain an average of three embryos per ovariole, making a total of about 54. However, many of the younger embryos were rounded up and degenerating (Fig. 3, II). One may conclude that although ovulation continues to some extent in the adult, virtually all the individuals which will subsequently be born are present as embryos in the teneral female.

The time of the initial ovulation was also determined by dissecting the largest embryos present in developing larvae. This event was found to occur in the 4th instar. Three generations are therefore represented in the 4th instar and adult: these individuals (grandparents) contain large embryos which will become parents; and the parent embryos themselves contain embryos which are destined to become the progeny of the parents. Such relationships are very similar to those first described in aphids by UICHANCO (1924).

Table 7 also includes information on the number of ovarioles present in *Megoura*. It will be seen that the number is not constant, and that unpaired ovarioles occur frequently. Of the 27 first and third instar larvae examined, twelve had the modal number of 18, five had 16, four had 17, five had 19, and one had 21.

The time relations of photoperiodic action

The period during the life of the parent when day length is effective in controlling the type of female progeny can readily be found by transferring long- or short-day parents to the antagonistic photoperiod at progressively later stages in larval development or adult life. The stages chosen were the third larval instar, the teneral adult immediately after the final moult, the adult immediately before parturition, and adults which have produced about 20 or 45 offspring respectively. At 15°C these stages are reached approximately 7, 14, 17, 20, and 25 days after the parent's birth.

Some representative progeny records are included in Table 8. Several points are worthy of comment. In the first place, it is clear that the function of the parent as an ovipara- or virginopara-producer can be switched in either direction, provided the change to the antagonistic photoperiod does not occur too late in the life of the parent. For example, third instar larvae transferred from a short to a long photoperiod produced a sequence of oviparae followed by virginoparae, whereas similar larvae transferred from a long to a short photoperiod produced a sequence of virginoparae followed by oviparae. In the case of the first instar larvae, however, all the progeny responded to the photoperiod their parent received after the first instar. Evidently the controlling mechanism had not yet become effective at this stage.

In the second place, there are clear signs that the timing of the reversal, as indicated by the numbers of female offspring of each type, advances steadily as the parent develops and her embryos mature and are born. Yet the point of reversal in the sequence of young undoubtedly differs according to the direction in which the photoperiod is changed. This matter is referred to in greater detail below.

A third feature brought out in Table 8 is that the reversal is abrupt and complete in the sense that mixed groups of oviparae and virginoparae do not occur after the transition has taken place. While this is in agreement with the view previously advanced regarding maternal control of embryonic development, it also points to the synchronous growth of embryos in different ovarioles. Thus it is reasonable to suppose that any determining stimulus would act on embryos of roughly the same

TABLE 8—TYPICAL PROGENY RECORDS OF INDIVIDUAL APTEROUS VIRGINOPARAE WHICH HAVE BEEN TRANSFERRED FROM A SHORT (12 HR) TO A LONG (16 HR) PHOTOPERIOD AND *vice versa* AT DIFFERENT STAGES IN LARVAL DEVELOPMENT AND ADULT LIFE

DIRECTION TRANSFERRED: FROM 12 TO 16 HR PHOTOPERIOD

Stage transferred	First instar larva			Third instar larva			Teneral adult			Parturition beginning			*ca.* 20 offspring born			*ca.* 45 offspring born		
Batch	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂
1	27				19			26			25			23			17	
2	26			16	6	1		7	3		28	13		30			13	8
3	10			11		11	1	10	9	10	3	3		15	15		31	2
4	16		6	14		8	22		1	18			16	4			23	
5	19		3	24			27			18			6				7	
Totals	98		9	65	25	20	50	43	13	46	56	16	22	72	15		91	10
Grand totals	107			110			106			118			109			101		

DIRECTION TRANSFERRED: FROM 16 TO 12 HR PHOTOPERIOD

Stage transferred	First instar larva			Third instar larva			Teneral adult			Parturition beginning			*ca.* 20 offspring born			*ca.* 45 offspring born		
Batch	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂	*V. ap.*	*Ov.*	♂
1		13		23			34			35			27			17		
2		21	3	25		1	21		2	16		3	20			26		1
3		20	4	5	5	4	17		7	26		9	16			15		3
4		22			21	3	8		8	14			16		6	22		7
5		23			16	1		17		14	3		19		3	11		
Totals		99	7	53	42	9	80	17	17	105	3	12	98		9	91		11
Grand totals	106			104			114			120			107			102		

Temperature 15°C, *V. ap.*, virginoparae apterae; *Ov.*, oviparae; ♂, males.

TABLE 9—THE PERCENTAGE AND NUMBERS OF FEMALE OFFSPRING THAT WERE INFLUENCED BY THE INITIAL PHOTOPERIOD WHEN THE LATTER WAS CHANGED DURING THE LARVAL DEVELOPMENT OR ADULT LIFE OF THE PARENT

Stage transferred	Days after birth of parents	Direction of change					
		From long to short photoperiod			From short to long photoperiod		
		No. of parents	Progeny		No. of parents	Progeny	
			Per cent virginoparae	No. of virginoparae + ♂♂		Per cent oviparae	No. of oviparae + ♂♂
First instar	0	37	0	0 + 0	25	0	0 + 0
Third instar	7	10	60	56 + 7	7	29	26 + 1
Teneral ♀	14	20	86	81 + 8	7	46	38 + 13
♀, parturition beginning	17	5	95	96 + 8	4	62	57 + 12
ca. 20 offspring born	20	—	—	—	4	75	67 + 9
ca. 45 off-spring born	25	—	—	—	9	100	102 + 6

VOL. 3 1959

growth stage. But any embryo so affected that was "held up" during later development might well be born among a group of offspring of the opposing type. This point is of some importance since it would seem that the order in which the progeny are born reflects with reasonable accuracy their respective ages. The positions they have previously occupied in the ovarioles, relative to the other offspring, can then be inferred with some confidence.

The progenies of all the parents subjected to the various treatments have been averaged in Table 9. Figures are given for the proportion of female embryos that have responded to the *initial* photoperiod at each stage of parental development. This is expressed as a percentage of the total female offspring. The absolute numbers of embryos influenced by the initial stimulus are also shown, together with the small but variable number of males.

When the aphids were transferred from a 16 hr to a 12 hr photoperiod in the third larval instar, 60 per cent of the female progeny became virginoparae in conformity with the initial treatment. The proportion of these forms rose to 86 per cent when the change of photoperiod was delayed until the final moult; and by the beginning of parturition only 5 per cent could still be influenced by the final short photoperiod. In terms of absolute numbers, an average of 56 female embryos responded when the initial long-day treatment extended from the first to the third instar, whilst 81 and 96 respectively did so when this treatment was continued up to the final moult and to the onset of parturition. Since there are usually 18 ovarioles, at least three of the four embryos which are present in each ovariole of the third instar larva seem destined to become virginoparae as a result of initial long-day treatment. In the teneral adult about five out of the six embryos are so committed; and three days afterwards the determination of embryos subsequently destined to be born is virtually complete. The inferred distribution of the embryos is shown diagrammatically in Fig. 4 (a, b). Male embryos, which have an expected frequency of less than one per ovariole (see p. 110), are omitted.

A different response was obtained when the short photoperiod preceded the long one (Table 9 and Fig. 4 c, d). Although the proportion of offspring developing as oviparae duly increased with the growth of the parent, the tempo of the progression was much less. Exposure of the first three instars to the short photoperiod resulted in an average production of only 26 oviparae, indicating that in the third instar larva one or sometimes two of the most advanced embryos could be regarded as future individuals of this type. No more than two or three embryos per ovariole were influenced when the short-day treatment was continued up to the final moult. And even up to the onset of parturition many ovarioles evidently had one or two small embryos that were still susceptible to the influence of the final long photoperiod. Possible reasons for this striking "asymmetry" in the response are considered later (p. 114).

Since many examples of this type of reversal were available for study, a number of progeny sequences were examined for the presence of forms intermediate between ovipara and virginopara. In *Acyrthosiphon* (KENTEN, 1955) and many other aphids, virginoparae with hind tibiae bearing "sensoria" or "scent plaques"—a normal

feature of the ovipara—are by no means rare. This abnormality was not found in *Megoura*, even in females born very close to the point of transition in the embryo sequence. Two oviparae with mixed reproductive systems containing both eggs and embryos were noted.

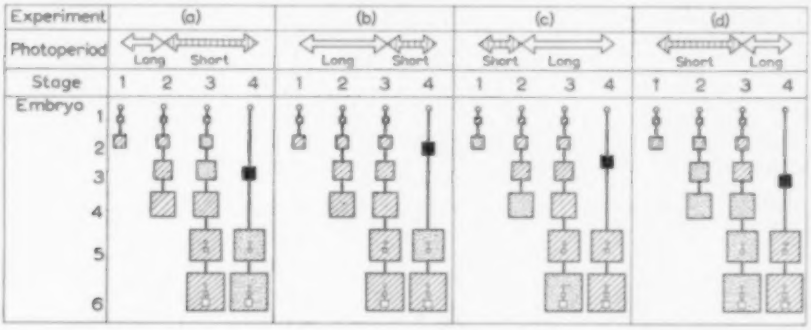


FIG. 4. The action of different photoperiodic régimes on the sequence of female embryos in the maternal ovarioles. Embryos destined to become virginoparae are indicated by shaded squares, future oviparae by stippled squares. Degenerating embryos are shown black. Note that aphids at growth stages 5 and 6 have already begun to form the embryos of the succeeding generation. Stages of development of the mother: (1) first instar larva; (2) third instar larva; (3) teneral adult; (4) female near end of reproductive life.

Nutrition

Experiments were also carried out to discover whether the nutritional condition of the host plant influenced form determination. Since the production of oviparae under natural conditions is confined to the late summer and autumn, it is worth enquiring whether the development of this form is favoured by deficiencies (or other changes) in the plant phloem sap. Potted broad beans (variety Sutton's Dwarf) were prepared for this purpose. The plants had flowered and set seed some weeks previously and when used had lost most of their foliage by abscission. The aphids colonized the stems, which still remained green in parts. They were exposed from the first instar to a 16 hr photoperiod at 15°C.

The resulting adult apterous virginoparae provided clear indications that the food supply was impoverished: for the mean weight of eight individuals was only 1·3 mg compared with a figure of 5·4 mg for parents reared on germinating beans. However, when these diminutive aphids were tested on similar bean plants they yielded an average of 28 apterous virginoparae and 2 males. No oviparae were produced. Malnutrition does not therefore appear to counteract the effects of a long day, as it may sometimes do in the induction of diapause (LEES, 1953).

It was conceivable, nevertheless, that the aphids would still be affected by physiological changes associated with the senescence of a perennial plant. Potted specimens of a common host, the vetch *Lathyrus pratensis*, which had stood outdoors throughout the summer, were used in September when signs of senescence

had become apparent in the foliage. The plants, together with the parent aphids, were again put on a 16 hr photoperiod at 15°C. All the individuals reared proved to be virginopara-producers thus providing no evidence that nutrition affects the production of oviparae.

Male determination

The rôle of temperature and photoperiod. Data on the numbers of males which occurred in the families of wingless virginoparae reared at different temperatures and photoperiods have been collected in Table 10. Some examples of individual progenies, which include information on male determination, have already appeared in Tables 1–3, 5–6, and 8.

TABLE 10—THE INFLUENCE OF TEMPERATURE AND PHOTOPERIOD ON THE PRODUCTION OF MALE PROGENY BY APTEROUS VIRGINOPARAE

Temperature (°C)	Photoperiod (hr light/24 hr)	No. of parents	No. of males per parent	
			Range	Mean ± S.D.
25	12	9	0–0	0
	16	9	0–0	0
20	12	27	5–21	13·7 ± 3·44
	16	44	0–21	8·0 ± 5·59
15	0	22	2–22	8·5 ± 6·04
	2	17	2–18	12·4 ± 3·92
	4	19	0–12	4·7 ± 3·04
	8	18	3–15	7·0 ± 2·91
	12	37	3–17	11·6 ± 3·02
	14·5	11	5–18	11·6 ± 5·13
	15	16	4–19	12·1 ± 4·31
	16	25	1–20	10·4 ± 4·40
	24	18	1–15	5·3 ± 3·94
11	12	19	0–5	2·5 ± 1·84
	16	32	0–9	2·9 ± 2·11

An inspection of the ranges given in Table 10 shows that the proportion of males produced by individual parents is strictly limited. Although these apterae gave birth to nearly 100 offspring each at medium temperatures, no treatment yielded more than 22 males. But below this upper limit the numbers were extremely variable. An analysis of variance* was therefore carried out with the following results.

Temperature was an influential factor. No males at all appeared at 25°C whereas at 20° and 15°C they were fairly abundant, the 254 parents yielding an overall mean of 9·6 males for all conditions of photoperiod. The frequency of males at these two latter temperatures was not, however, significantly different at the 5 per cent level. Significantly fewer males occurred at 11°C, the overall mean being 2·7. From these results it seems that there is an optimal thermal range for male production which lies in the 15°–20°C region. Higher or lower temperatures suppress the appearance of this form to a greater or lesser extent.

* Kindly undertaken by the Staff of the Statistical Laboratory, Cambridge.

Rather surprisingly, photoperiod had no detectable influence on male determination. Results were available for a complete series of photoperiods at 15°C and for typical long and short day lengths at 20° and 11°C. But none of the means differed significantly (Table 10).

A feature of these observations was the high male variance. Even with parents that had been reared together under apparently identical conditions the numbers sometimes fluctuated between 0 and 21 (Table 10). An attempt was made to trace the source of this variability. If the cause was genetic, due to the appearance of dominant mutations in the clone, it should have been possible to select lines with high and low male production. Such an experiment was begun with two apterous virginoparae, one of which had yielded 13 males and the other three. Two daughter virginoparae were selected at random and these in turn gave rise to a number of females (grand-daughters of the original pair) which were put on a 12 hr photoperiod at 15°C. The females from the "high male" lineage averaged 11·3 males, those from the "low male" lineage 11·8.

VOL. 3 1959

A further possibility was that temperature fluctuations were responsible. Some disturbance in temperature was unavoidable during the short periods when the cabinets were opened for watering or changing the plants. Some weight is lent to this suggestion by the evidence of SHIBATA (1934, 1952, 1954), who found that male production in *Myzocallis kuricola* was stimulated by fluctuating temperatures. The most favourable type of daily cycle consisted of a constant low temperature (15°C) period of one or more hours alternating with a constant high temperature (25°C). This experiment was repeated with *Megoura*. The treatment involved a 2 hr period at 14°C (which coincided with the beginning of the 16 hr light phase), followed by 22 hr at a constant temperature of 20°C. This daily régime was begun a few days before the birth of the parent virginoparae and was continued throughout larval and adult life. Male production by these parents was compared with that of a parallel series reared at a constantly maintained temperature of 20°C. In the latter instance special precautions were taken to provide as complete a thermal constancy as possible.

The series of 15 parents reared under constant conditions produced a mean number of 10·5 males; the 17 parents from the fluctuating temperature yielded a mean of 7·9 males. The value for t does not reach significance at the 5 per cent level.

The position of males in the progeny sequence

The individual progeny records given in Tables 1–3, 5–6, and 8 suggest that male offspring do not occur with equal frequency at different times in the reproductive life of the parent. This is borne out when the numbers of males present in the five serial batches are plotted in chart form (Fig. 5). Since the intention of collecting the young in groups of 20 (p. 94) could only be realized approximately, some selection of the available records has been necessary. The results illustrated are for parents that yielded five successive batches of offspring, the number per batch falling within the arbitrary limits of 13–27. These 40 parents had all been reared at 15°C although in different photoperiods.

The progeny totals averaged 19·0, 19·5, 20·5, 20·3, and 18·6 respectively, while the mean numbers of males in the corresponding batches were 0·0, 2·1, 5·1, 3·2, and 0·1. In other words, no males occur in the first 20 or so offspring; the incidence then rises to a maximum in the middle of the progeny sequence, but again declines almost to zero towards the end of reproduction. Other observations in which the parent aphids were only transferred two or three times indicated that the males in the second of the five collections must have been among the last to be born.

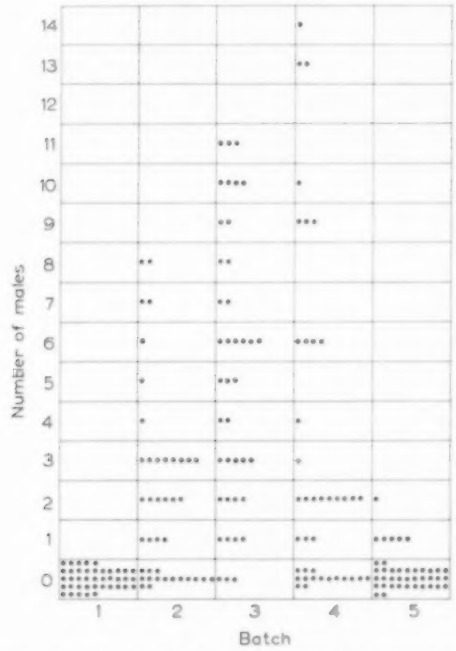


Fig. 5. The incidence of males in the five successive batches of young collected from 40 parent virginoparae. Temperature, 15° C.

In terms of the embryo sequence this suggests that the first two embryos in the ovariole, representing a total progeny of $2 \times 18 = 36$ individuals, are almost invariably female regardless of the environmental circumstances. The highest frequency of males should be found among the third and fourth embryos in the sequence, whereas a much lower frequency obtains in the fifth and sixth. If the male embryos are distributed evenly between the ovarioles, there would usually be less than one in each since the total complement of males does not often exceed the ovariole number of 18.

DISCUSSION

The control of polymorphism in long-established clones of *Megoura* appears to depend upon a series of maternal "switch" mechanisms which direct and

canalize the multiple potentialities of the parthenogenetic oöcytes and embryos. It is these mechanisms that are responsive to the environment. The embryos, in their turn, acquire and retain their sensitivity to the successive maternal signals only during certain limited growth periods. The competent embryos are then capable of following different developmental pathways which in the present instance are represented by simple pairs of alternatives. According to this view, the production of the four possible forms by the aptera involves three such decisions which arise in succession. The first results in the determination of sex; the second in the differentiation of female embryos as virginoparae or oviparae; and the third in the differentiation of virginoparae as winged or wingless individuals. We are concerned with the first two of these events in the present paper.

The early studies of MORGAN (1909, 1910, 1915), VON BAEHR (1908, 1909), and others on the aphid caryotype showed that, whereas in a given species the chromosome number of all the female forms was the same, the male possessed one chromosome less. It has therefore been generally accepted that sex is determined by the chromosomes, the mechanism being of the common XX–XO pattern (LAWSON, 1936; WHITE, 1954). SHIBATA (1954) has recently been inclined to doubt this classical interpretation since his researches on the primitive species *Myzocallis kuricola* have shown that the chromosome number is alike in the two sexes ($2n = 12$). However, the possibility that the second sex element in the male is a Y chromosome with extensive euchromatic regions does not seem to have been entirely excluded.

The cytology of the parthenogenetic eggs has been studied by MORGAN (1910) and LAWSON (1936). And a particularly clear account of the maturation of both female and male eggs has been given by SCHWARTZ (1932) for *Tetraneura ulmi*. It is generally agreed that in this type of diploid parthenogenesis the eggs undergo only one division, a single polar body being formed. In the female egg the chromosomes go through what is virtually a normal mitosis, having already split by early prophase. In the male egg, however, the X chromosomes differ from the autosomes in that they enter prophase in the undivided condition. They proceed to pair at zygotene but eventually fall apart without division or chiasma formation. One X chromosome then passes into the polar body.

If the orthodox view of sex determination is applicable to *Megoura* (for which no cytological data are yet available), the critical events which affect the behaviour of the X chromosome must take place in the oöcyte some time before cleavage begins. The site of these events is probably the germarium, since this organ contains the nurse cells, oögonia, and enlarging oöcytes (UICHANCO, 1924; LAWSON, 1936). Thus the sex will no doubt have been established by the time the oöcyte is liberated into the ovariole.

If the sex of the parthenogenetic egg is decided genetically, it becomes desirable to link this event with the highly abnormal sex ratio and with its modification by the environment. One possibility is that the deficiency in males is due to differential mortality. If male and female eggs are ovulated in equal numbers, mortality should reach 50 per cent among the early ova or embryos, since this phase of embryogenesis produces no males at all. In fact, embryos formed at this time

very rarely show signs of degeneration (rounding up, followed by gradual resorption as the embryo passes down the ovariole), although the regression of small embryos or, more especially, oöcytes would obviously be difficult to detect. It is therefore felt that attention should be directed instead towards the possibility that the chromosomal behaviour is itself influenced either indirectly through the maternal physiology or directly by the environment.

Whatever the mechanism of determining the sex ratio may be, it is clear that two different components should be distinguished. The fact that the proportion of males has never been made to exceed one quarter of the total progeny suggests that the maternal reproductive system itself has an important regulatory function. And the definite "pattern" of male production in the sequence of offspring is surely indicative of the same property. While preliminary experiments have suggested that this pattern could be slightly shifted by different temperature manipulations, the occurrence of males in the middle of the sequence remains a very constant feature. Yet, as it is most unlikely that the sequence is fixed in any absolute sense, there would appear to be some homeostatic mechanism within the germarium, or between different germaria, whereby the chances of a given egg becoming a male is adjusted according to the incidence of males among previous eggs.

The second component in the control of male production is environmental. Although it is impossible by any treatment yet devised to increase the proportion of males beyond a certain point, their formation can readily be inhibited. In this respect high temperature is particularly effective. Many other aphids, for example, *Aphis chloris* (Wilson, 1938), *Myzus persicae*, *Brevicoryne brassicae* (Bonnemaison, 1951), and *Acyrthosiphon pisum* (Kenten, 1955) respond to temperature in a similar manner. In *Acyrthosiphon* and *Myzocallis kuricola* (Shibata, 1934) male production is again markedly reduced at temperatures below the 15°–20°C range. This effect, which is also noticeable in *Megoura*, does not appear to be merely a consequence of the lowered fecundity.

Photoperiod is without appreciable influence on the frequency of males in *Megoura*. This response is quite unlike that in other aphids. In *Acyrthosiphon*, for example, no males at all occur in long days at any temperature (Kenten, 1955), whereas in *Myzus* and *Brevicoryne* they are formed in reduced numbers (Bonnemaison, 1951).

If these factors indeed control the sex ratio through the chromosomes, how is this control exercised? Since it is well known that abnormal temperatures can cause pairing failure with resulting chromosome loss in other material (Darlington, 1939), the evolution of a caryotype responding differentially to temperature within the normal biological range could be readily envisaged. Such an explanation might suffice for *Megoura*. However, it is difficult to believe that light also could operate directly in species with a photoperiodically-controlled male determination. Since there are indications that the development of the female egg into ovipara or virginopara may be controlled by humoral means (see below), a possible hypothesis is that the type of endocrine balance that favours the production of oviparae also promotes

the production of male eggs. However, this view is not altogether satisfactory as KENTEN (1955) has shown that the combinations of photoperiod and temperature that most favour these respective forms are by no means identical.

Turning now to the differentiation of the female embryos, there seems little reason to doubt that their condition as future oviparae or virginoparae is decided *after* the determination of sex. Thus, although the first two embryos in each ovariole of the first instar larva are presumably determined for sex (they are almost invariably female), they can be caused to develop into either type of female by varying the photoperiod and temperature during the early larval development of the parent. The present experiments throw some light on the mechanism and timing of the controlling process.

The nature of the response to "intermediate" environmental conditions has already been stressed. Critical photoperiods at moderate temperature, or short photoperiods partially counteracted by a high temperature, provide conditions of this sort. Now, as the body wall and blood of *Megoura* (a green aphid) are highly translucent, photoperiod (and, of course, temperature) could be acting directly on the developing embryos. In these circumstances we should expect aphids reared under the intermediate conditions just mentioned to produce offspring of both types in random sequence. This never occurs. If the alternative is true, and the form of the offspring is regulated by the maternal physiology, the element of randomness should apply to the parents, some individuals becoming ovipara-producers and others virginopara-producers. Although this theoretical result is sometimes achieved, the families usually consisted of alternating groups of virginoparae and oviparae. Nevertheless, it is equally difficult to account for this result unless the form determination is under maternal control.

This phenomenon has not so far been noted in other species of aphids. The production of families which included both virginoparae and oviparae has often been observed; indeed some authors have recorded *only* mixed progenies—a circumstance that may have been caused by the selection of temperatures which were unsuitable for the most striking demonstration of the photoperiodic effect. Nevertheless, since in these experiments the parent aphids were not usually isolated during their reproductive period, it would be impossible to recognize any regularities of the type described above. The few individual progeny records published by BONNEMAISON (1951) and KENTEN (1955) are not inconsistent with these findings, although the comparatively low fecundity of the species used, and the preponderance of males in some families, renders the data more difficult to interpret.

Little is known at present regarding the physiological mechanism in the parent which controls the type of female offspring. However, it is worth calling attention to the similarities with the photo-induction of diapause. The "intermediate" nature of the response to permanent darkness is especially striking. And in addition, the type of summation which is observed with long days and high temperatures on the one hand, and with short days and low temperatures on the other, is a familiar phenomenon in many insects with an environmentally controlled diapause (LEES, 1955).

VOL. 3 1959

Some inferences are possible concerning the final stage of the governing mechanism. This may consist of two components: the alternative maternal stimuli—possibly humoral in nature—and the embryo itself which certainly differs in its sensitivity to these stimuli according to its stage of growth. In a constant photoperiod it seems probable that the appropriate maternal stimulus is supplied continuously throughout larval development and perhaps adult life. All the embryos therefore have an opportunity of responding to it. This is not the case, however, when the photoperiod is reversed during the late larval development of the parent. In these circumstances the older embryos have already passed the stage of competence and have become refractory to the second stimulus. In addition, there are also indications that the youngest embryos are also insensitive. For example, Fig. 4(a) shows that when a long photoperiod is given during the first three larval instars, the fourth and smallest embryo is unaffected; and two or three embryos fail to respond to an initial short photoperiod (Fig. 4c). Finally, under "intermediate" but apparently constant conditions the controlling system may perhaps be regarded as in unstable balance. This is readily upset by slight fluctuations in the internal or external environment, and the embryos then react accordingly.

Fig. 4 shows that the determination of embryos as virginoparae, in response to an initial long photoperiod, appears to proceed more rapidly than the determination of embryos as oviparae by an initial short photoperoid. This "asymmetry" in the response is more difficult to explain. One possibility is that both female morphs are controlled by a single stimulus, the type of individual depending on whether the stimulus is present or absent. The possible mode of action of such a system during the period of maternal development when the number of embryos per ovariole is increasing can be appreciated from an inspection of Fig. 4. Clearly, if the stimulus is available for a given period, the embryos which will pass through the reactive stage (probably in positions 2 or 3) will outnumber those that have reached a refractory condition (perhaps in stage 4). The latter embryos will become females of the opposing type if the stimulus is absent during the same period.

Other possibilities also exist. It will be recalled that the first two embryos in the ovarioles of newly born larvae can be converted into either virginoparae or oviparae by post-natal photoperiodic treatment. This might indicate either that they were not competent to respond to the maternal stimulus, or that the latter was not yet available, or both. The evidence mentioned above suggests that the first alternative is correct; and in this event it may well be that the controlling mechanism starts to operate before the birth of the parent. In the present experiments all grandparents were exposed to long day conditions in the stock cultures. It is possible that the mechanism, once functioning as a virginopara-determiner, displays a certain resistance to reversal by a short photoperiod. Further work is required to settle this point.

Some reference must finally be made to the classification of aphid forms. The term "sexupara" has been purposely avoided in this paper. Many systematists employ the term merely to denote a producer of sexual forms (gynopara = ovipara

producer; andropara = male producer). This usage is misleading. In the first place, there is some danger that the "sexupara" will be thought of as a distinct form in all aphids. Yet in a species without host alternation, such as *Megoura*, the sexupara is the *same individual* as the virginopara. Indeed, a female *Megoura*, reared in the appropriate environment, might be judged to be first a virginopara, then an "andropara" and finally a "gynopara"! Even more complex terminologies have sometimes been devised for these "ecological forms" (e.g. SHIBATA, 1954). There must nevertheless be a strong suspicion that in many monoecious aphids all these "forms" are identical and that the type of progeny reflects the various potentialities of embryonic development under particular environmental conditions. However, an additional and more important source of confusion relates to the fact that in species with host alternation the sexupara is undoubtedly a distinct entity. For example, it is well known that in *Aphis fabae* the winged viviparous females (gynoparae) that return to the primary host in autumn differ both morphologically and in their behaviour from alate virginoparae (KENNEDY and BOOTH, 1954).

VOL.
3
1959

The differences between alate virginoparae and gynoparae, although definite, are comparatively subtle. It is possible that the failure to make this distinction may be responsible for a further apparent anomaly in the literature. From his extensive observations on *Macrosiphum solanifolii* (= *M. euphorbiae*) SHULL (1928, 1929) concluded that wing determination was under the control of photoperiod. In his experiments short days were shown to favour the appearance of "alatae" (that is, by implication, alate virginoparae), whereas long days favoured apterous virginoparae. Yet subsequent authors have invariably reported that photoperiod has little or no effect on wing production—a finding which is confirmed in *Megoura*. Nevertheless, it is striking that the curves for photoinduction of "alatae" in *Macrosiphum* and of oviparae in *Megoura* are almost identical, the resemblance even extending to the response under "intermediate" conditions of permanent darkness. As BONNEMAISON (1951) suggests, it would appear likely that SHULL's "alatae" were in reality gynoparae.

SUMMARY

(1) Apterous virginoparae from long-established clones of *Megoura* are capable of producing three types of daughters (parthenogenetic winged and wingless virginoparae and sexual oviparae) in addition to males.

(2) This polymorphism is primarily controlled by the environment acting through a series of maternal "switch mechanisms". The latter in turn direct the growing oöcytes and embryos into different alternative developmental pathways. The first dichotomy, probably at the oöcyte stage, decides the sex; the second, which affects partly grown female embryos, results in their differentiation as oviparae or virginoparae.

(3) Although sex is doubtless determined genetically, an over-riding physiological control is exercised both by the maternal reproductive system itself and by the external environment. The intrinsic regulatory properties of the former are

indicated by the strict limitations imposed on the numbers of males formed by the germaria; and by the fact that males are virtually confined to the middle of the sequence of offspring. Environmental control is exemplified by the complete suppression of males at high temperature and by their partial suppression at low ones. But photoperiod is without influence.

(4) The differentiation of female embryos as oviparae or virginoparae is wholly governed by photoperiod and temperature. High temperatures and long photoperiods promote the production of virginoparae, while the opposing conditions favour oviparae. At moderate temperatures (*ca.* 15°C) all the female offspring are of one type or the other depending on the photoperiod.

(5) Critical cycles of illumination (for example, a photoperiod of $14\frac{1}{2}$ hr or permanent darkness at 15°C) induce some parents to give birth to virginoparae only, whereas others yield oviparae only. Although the majority have mixed progenies under these conditions, the two forms are not distributed at random in the reproductive sequence but occur in alternating groups. These findings suggest that light and temperature act primarily on the parent. Embryonic determination may be mediated by humoral means.

(6) Determination of the oldest embryos as oviparae or virginoparae begins during the early larval instars of the mother. Whilst in virginopara-producers this process is almost complete by the final moult, in ovipara-producers it continues until much later in reproductive life. The progression of determination can be explained in terms of the competence of embryos in different stages of growth to respond to alternative maternal stimuli.

Acknowledgements—I am greatly indebted to my colleague Dr. J. W. L. BEAMENT for designing the plant growth cabinets used in this work. Mr. M. J. WAY of the Department of Insecticides and Fungicides, Rothamsted Experimental Station, kindly drew my attention to the favourable nature of the aphid material and contributed many valuable suggestions in a discussion of the results. My warmest thanks are also due to Mr. H. L. G. STROYAN for advice on matters of aphid biology and taxonomy, and to Mr. M. J. WELLS for drawing Fig. 1.

REFERENCES

BAEHR W. B. VON (1908) Über die Bildung der Sexualzellen bei Aphididae. *Zool. Anz.* **33**, 507–517.

BAEHR W. B. VON (1909) Die Oögenese bei einigen viviparen Aphididen und die Spermatogenese von *Aphis saliceti*, mit besonderer Berücksichtigung der Chromatinverhältnisse. *Arch. Zellforsch.* **3**, 269–333.

BONNEMAISON L. (1951) Contribution a l'étude des facteurs provoquant l'apparition des formes ailées et sexuées chez les Aphidinae. *Ann. Épiphyt.* (C) **2**, 1–380.

DARLINGTON C. D. (1939). *The Evolution of Genetic Systems.* Cambridge University Press.

DAVIDSON J. (1929) On the occurrence of parthenogenetic and sexual forms in *Aphis rumicis* L., with special reference to the influence of environmental factors. *Ann. appl. Biol.* **16**, 104–134.

DE FLUITER H. J. (1950) De invloed van daglengte en temperatuur op het optreden van de gelachtsdieren dij *Aphis fabae* Scop., de zwarte bonenluis. *Tijdschr. PlZiekt.* **56**, 265–285.

KENNEDY J. S. and BOOTH C. O. (1954) Host alternation in *Aphis fabae* Scop. II Changes in the aphids. *Ann. appl. Biol.* **41**, 88–106.

KENTEN J. (1955) The effect of photoperiod and temperature on reproduction in *Acyrthosiphon pisum* (Harris) and on the forms produced. *Bull. ent. Res.* **46**, 599–624.

LAWSON C. A. (1936) A chromosome study of the aphid *Macrosiphum solanifolii*. *Biol. Bull., Woods Hole* **70**, 288–307.

LEES A. D. (1953) Environmental factors controlling the evocation and termination of diapause in the fruit tree red spider mite *Metatetranychus ulmi* Koch (Acarina: Tetranychidae). *Ann. appl. Biol.* **40**, 449–486.

LEES A. D. (1955) *The Physiology of Diapause in Arthropods*. Cambridge University Press.

LICKERISH L. A. (1953) Studies on commercial octamethylpyrophosphoramide (Schradan)—V. Insecticidal comparisons of the two main constituents. *J. Sci. Fd Agric.* **4**, 24–28.

MARCOVITCH S. (1924) The migration of the Aphididae and the appearance of the sexual forms as affected by the relative length of daily light exposure. *J. agric. Res.* **27**, 513–522.

MORGAN T. H. (1909) Sex determination and parthenogenesis in phylloxerans and aphids. *Science* **29**, 234–237.

MORGAN T. H. (1910) The chromosomes in the parthenogenetic and sexual eggs of phylloxerans and aphids. *Proc. Soc. exp. Biol., N.Y.* **7**, 161–162.

MORGAN T. H. (1915) The predetermination of sex in phylloxerans and aphids. *J. exp. Zool.* **19**, 285–316.

SCHWARTZ H. (1932) Der Chromosomzyklus von *Tetraneura ulmi* de Geer. *Z. Zellforsch.* **15**, 645–687.

SHIBATA B. (1934) Ecological studies of plant lice—III. Experimental production of amphigonous females and males. *Bull. Utsonomiya Agric. Coll.* **5**, 177–207.

SHIBATA B. (1952) Ecological studies of plant lice—VI. With reference to experimental production of males. *Bull. Utsonomiya Agric. Coll.* (N.S.) **1**, 307–318.

SHIBATA B. (1954) Ecological and cytological studies of an aphid *Myzocallis kuricola* Mats. *Bull. Utsonomiya agric. Coll.* (N.S.) **2**, 101–167.

SHULL A. F. (1928) Duration of light and the wings of *Macrosiphum solanifolii*. *Arch. EntwMech. Org.* **113**, 210–239.

SHULL A. F. (1929) The effect of intensity and duration of light and darkness, partly modified by temperature, upon wing production in aphids. *Arch. EntwMech. Org.* **115**, 825–851.

SHULL A. F. (1930) Control of gamic and parthenogenetic reproduction in winged aphids by temperature and light. *Zeit. indukt. Abstamm.-u. VererbLehre* **55**, 108–126.

UICHANCO L. B. (1924) Studies on the embryology and postnatal development of the Aphididae with special reference to the "symbiotic organ" or "mycetome". *Philippine J. of Science* **24**, 143–247.

WHITE M. J. D. (1954) *Animal Cytology and Evolution*. 2nd ed. Cambridge University Press.

WILSON F. (1938) Some experiments on the influence of environment upon the forms of *Aphis chloris* Koch (Aphididae). *Trans. R. ent. Soc. Lond.* **87**, 165–180.

VOL. 3 1959

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 118 *to* 119. *Pergamon Press Ltd., London. Printed in Great Britain*

SUSTAINED FLIGHT IN PHORMIA (BY A NEW METHOD) AND ITS EFFECT ON BLOOD pH

STANLEY FRIEDMAN*

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service
U.S. Department of Health, Education, and Welfare, Bethesda, Maryland

(*Received* 24 *July* 1958)

Abstract—Sustained flight of 120 min or more has been obtained with *Phormia regina* Meig. Blood pH's measured before and after flight appear to fall within the same range (6·9–7·1).

THE utilization of *Phormia* for metabolic studies has occasioned the need for a simple method of promoting sustained activity such as flight. Our attempts to utilize flight-provoking techniques already devised for Diptera have met with little success. The methods of CHADWICK and GILMOUR (1940) for *Drosophila* and of HUDSON (1958) for *Phormia* both depend upon repetitive stimulation of the tarsal reflex for inducing flight, and fail in their purpose in *Phormia* as a result of an apparent adaption of the insects to the stimulus. Thus, "long" flights using these methods are discontinuous and subject to a great many rest periods. Supplementation of the devices with light and air currents lead to insignificant increases in flight duration.

The method described here derives from two observations previously recorded in the literature. Firstly, that some insects will, if the tarsi are amputated, fly to exhaustion (FRAENKEL, 1932) and secondly, that the stimulus for continued flight in some specimens is a continuous exposure to a current of air (CHADWICK, 1953). Combining these two stimuli has led to steady flights of 2 hr or more with subsequent continued attempts in spite of obvious exhaustion. The flights could be terminated by stopping the air flow or by permitting the animals to stand on a flat surface. The apparatus used consists simply of a wind tunnel made of a piece of glass tubing 30 in. long and 3 in. in diameter at the end of which is placed the intake of a motor-driven "snail type" blower (Aminco 4-690A). A glass rod which may be easily removed is fastened along the top inside of the tube. Each fly is suspended from the glass rod by a 1½ in. length of thread attached to its scutum with paraffin or Duco cement. The flies are prepared for mounting by removing the tarsus of each leg close to the tarso-tibial articulation. This may be done rapidly without microscopic examination, since there appears to be no significant difference in attitude or activity as a result of indiscriminate cutting. To all outward appearances there is no blood lost as a result of the operation.

* Present address: Department of Entomology, Purdue University, Lafayette, Indiana.

Using the above method, it has been possible to determine blood pH on individual flies after sustained flight activity. Blood pH was measured with a Beckmann Model G meter equipped with a modified micro glass electrode. The electrode was drawn from Corning 015 glass tubing, its total capacity maintained at 1·3–1·5 μl, and wall resistance at less than 2 MΩ. From five-day old flies randomly withdrawn from a rearing cage, values of 6·96±0.05 (eight individuals) were obtained, whereas the pH's of others withdrawn from the same cage and flown continuously for an average of 100–120 min were 6·99±0·1 (six individuals) when the blood was removed and measured immediately after flight. It is apparent from these figures that either no acid products of metabolism are released into the blood or the buffering capacity of the blood is great enough to sustain the pH at its normal value in spite of any outpouring of organic acid into it during sustained activity.

REFERENCES

CHADWICK L. E. (1953) The flight muscles and their control. In Roeder, K. D. (ed.) *Insect Physiology*. John Wiley, New York.

CHADWICK L. E. and GILMOUR D. (1940) Respiration during flight in *Drosophila repleta* Wollaston: The oxygen consumption considered in relation to the wing rate. *Physiol. Zool.* **13**, 398–410.

FRAENKEL G. (1932) Untersuchungen über die Koordination von Reflexen und Automatisch-Nervösen Rhythmen bei Insekten. I. Die Flugreflexe der Insekte und Ihre Koordination. *Z. Vergl. Physiol.* **16**, 371–393.

HUDSON A. (1958) The effect of flight on the taste threshold and carbohydrate utilization of *Phormia regina* Meig. *J. Ins. Physiol.* **1**, 293–304.

VOL. 3 1959

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 120 *to* 124. *Pergamon Press Ltd., London. Printed in Great Britain*

EXPERIMENTS ON THE *ISO*XANTHOPTERIN METABOLISM IN *DROSOPHILA MELANOGASTER*

G. E. GRAF,* E. HADORN, and H. URSPRUNG

Institute of Zoology and Comparative Anatomy, University of Zürich, Switzerland

(*Received* 28 *July* 1958)

Abstract—The enzyme xanthine oxydase (or xanthine dehydrogenase) converts 2-amino-4-hydroxypteridine to *iso*xanthopterin. The mutant *rosy* (*ry*) does not form *iso*xanthopterin, most likely due to the absence of the enzyme. *ry* flies contain more 2-amino-4-hydroxypteridine than do wildtype, with males accumulating more than females. Injection of 2-amino-4-hydroxypteridine into wildtype larvae leads to an increase of *iso*xanthopterin over the control level, both in larval and pupal stages. Injection of the same compound into *ry* larvae did not lead to the formation of *iso*-xanthopterin. Similar negative results were obtained with a number of other pteridines, whereas the injection of wildtype Malpighian tubes into *ry* clearly causes the appearance of *iso*xanthopterin. Malpighian tubes treated with ultra-violet light caused only a small increase over the *ry* controls. Wildtype flies contain more drosopterines than do *ry*. We were, however, not successful in increasing the drosopterine content in *ry* by means of injection of various other pteridines. Attempts to phenocopy the *ry* gene through treatment with 2-amino-4-hydroxypteridine-6-aldehyde were unsuccessful.

INTRODUCTION

THE mutant ry^2 of *Drosophila melanogaster* does not appear to form *iso*xanthopterin at any stage of its development (HADORN and SCHWINCK, 1956). This is most likely due to the absence of the enzyme xanthine oxydase (or xanthine dehydrogenase), responsible for the conversion of 2-amino-4-hydroxypteridine (HB_1) to *iso*xanthopterin; it is remarkable that a deficiency in the same enzyme was described in the mutant *maroon-like* (FORREST *et al.*, 1956; HADORN, 1956; GLASSMAN and MITCHELL, 1958). The ry^2 phenotype is also characterized by an increase in sepia pterin and HB-pterins (HADORN and SCHWINCK, 1956; HADORN and GRAF, 1958). The pteridine metabolism of ry^2 has been demonstrated to be non-autonomous in the eyes (HADORN and SCHWINCK, 1956) and also in the testes (HADORN *et al.*, 1958). While it is clear that ry^2 has increased amounts of the HB-fraction, this spot contains in addition to HB_1 a second compound HB_2 which is probably biopterin (FORREST and MITCHELL, 1955). Therefore it appeared to be of some interest to ascertain if 2-amino-4-hydroxypteridine accumulates significantly in ry^2. Further, the *in vivo* conversion of 2-amino-4-hydroxypteridine to *iso*xanthopterin in wildtype *Drosophila melanogaster* was investigated. FORREST *et al.* (1956) found that flies of the genotype *white-apricot* fed 2-amino-4-hydroxypteridine as larvae contained *iso*xanthopterin as adults where ordinarily none could be

* Aided by a postdoctoral fellowship of the American Cancer Society.

demonstrated. Finally, in view of the marked influence of transplanted wildtype Malpighian tubes and fat bodies on the pteridine content of ry^2 (HADORN and SCHWINCK, 1956; HADORN and GRAF, 1958), experiments were carried out in an attempt to gain further insight into the modification of the ry^2 phenotype.

The 2-amino-4-hydroxypteridine content of three days old ry^2 and wildtype (strain Sevelen) imagos were compared. Thirty flies of each genotype were squashed directly on the same sheet of Whatman No. 1 paper and chromatographed in propanol—1 per cent ammonia (2 : 1). The blue fluorescing HB spot was located with a long wavelength UV lamp and eluted with dilute ammonia. The eluate was rechromatographed in 5 per cent acetic acid which has been shown to separate the

TABLE 1—ARBITRARY FLUORESCENT UNITS OF 2-AMINO-4-HYDROXYPTERIDINE IN 3 DAYS OLD IMAGINES. MATERIAL FROM 30 INDIVIDUALS WAS USED PER DETERMINATION (n)

	n	Wild type (+/+)	Rosy^2 (ry^2/ry^2)
males	6	40·4 ± 6·0	100·0 ± 8·7
females	6	32·9 ± 4·7	47·6 ± 5·0

two constituents of this spot (FORREST and MITCHELL, 1955). The spot corresponding in R_f and fluorescence with synthetic 2-amino-4-hydroxypteridine was measured directly on the paper by means of the method of HADORN and KÜHN (1953). The results of these determinations are shown in Table 1.

It is clear that the mutant ry^2 accumulates 2-amino-4-hydroxypteridine, with males accumulating much more than females. This sex difference parallels the long known sex difference with regard to *iso*xanthopterin in wildtype demonstrated by HADORN and MITCHELL (1951). The difference is mainly due to the high concentration of this compound in the testes. Thus the proposed precursor of *iso*xanthopterin accumulates in ry^2 and shows the same sex difference in ry^2 that *iso*xanthopterin shows in wildtype.

To examine the *in vivo* conversion of 2-amino-4-hydroxypteridine to *iso*-xanthopterin, experiments were carried out in which Holtfreter solution saturated with the former compound was injected into third instar wildtype larvae. Injected larvae and uninjected controls of the same age were compared with regard to *iso*xanthopterin content at various intervals after injection. Chromatograms of larvae and pupae squashed directly on the paper were developed and measured as above. The data, shown in Table 2, indicate that individuals injected with the supposed precursor consistently contained higher amounts of *iso*xanthopterin than did controls. Injection of the same compound into ry^2 larvae did not lead to the formation of *iso*xanthopterin.

The possible action on the pteridine metabolism of ry^2 of a number of other pteridines, which have been reported as present in *Drosophila melanogaster*, was

also investigated. Synthetic samples of the pteridines used in the work reported in this place were kindly provided by Professor M. VISCONTINI of the Chemistry Institute of the University of Zürich. All experiments were carried out with ry^2, as described above for the 2-amino-4-hydroxypteridine injection experiments. Measurements were also made of adults less than 12 hr after emergence.—The injection of *iso*xanthopterin is of special interest since it might be postulated that the decrease of drosopterines in ry^2 is due to the absence of *iso*xanthopterin. Actually the injection of this compound did not lead to increased drosopterines. In a typical experiment, 24 controls had a mean of $10{\cdot}1 \pm 0{\cdot}3$ arbitrary fluorescent units of drosopterines while an equal number which had been injected with

TABLE 2—*Iso*XANTHOPTERIN IN WILDTYPE INDIVIDUALS INJECTED WITH 2-AMINO-4-HYDROXYPTERIDINE. DATA ARE EXPRESSED IN TERMS OF ARBITRARY UNITS OF FLUORESCENCE PER INDIVIDUAL

Hr after injection	n	Stage	*Iso*xanthopterin	
			Control	Injected
0–2	5	larvae	$6{\cdot}2 \pm 0{\cdot}6$	$8{\cdot}3 \pm 0{\cdot}6$
12–20	4	larvae	$16{\cdot}6 \pm 1{\cdot}8$	$25{\cdot}7 \pm 3{\cdot}1$
24	24	pupae	$54{\cdot}3 \pm 5{\cdot}3$	$72{\cdot}5 \pm 3{\cdot}9$

*iso*xanthopterin had a mean of $9{\cdot}6 \pm 0{\cdot}3$. However, the results do not exclude the possibility that *iso*xanthopterin was present in insufficient quantities to cause a significant increase in drosopterines, or more probably that it never reached the actual sites of drosopterin synthesis. Similar negative results were obtained with injected 2-amino-4-hydroxypteridine, 2-amino-4-hydroxpteridine-6-carboxylic acid, and xanthopterin. None of these compounds caused the appearance of detectable amounts of *iso*xanthopterin. Thus the pteridine pattern of ry^2 has still not been demonstrably altered by the injection of anything other than living tissue. The effect of temperature has been discussed by HADORN and GRAF (1958) and is assumed to differ fundamentally from the action of ry^+ tissues, since no *iso*-xanthopterin is formed. The results are in harmony with the hypothesis suggested by HADORN and GRAF (1958) that only the injection of a source of the enzyme xanthine dehydrogenase causes the appearance of *iso*xanthopterin in ry^2.

In another series of experiments Malpighian tubes from wildtype larvae were irradiated with ultraviolet light for 7 min before being injected into female ry^2 larvae. The technique is described in more detail by URSPRUNG *et al.* (1958), who found that such treatment leads to morphological changes clearly discernible after about 30 hr and ultimately to the death of the transplanted tubes. Flies containing such treated tubes as well as others with untreated Malpighian tubes, along with wildtype and ry^2 controls, were decapitated and the heads chromatographed as above. The flies were one to two days old. The results of the

fluorescence measurements are shown in Table 3. As has been demonstrated repeatedly (see HADORN and GRAF, 1958), the addition of untreated wildtype Malpighian tubes to ry^2 caused a clear increase in drosopterines as well as the appearance of *iso*xanthopterin and two other compounds one of which is probably xanthopterin (ZIEGLER-GÜNDER and HADORN, 1958). The tubes treated with ultra-violet light caused only a very small increase over the ry^2 controls. As determined by a t-test, the difference was not significant at the 5 per cent level. Some of these heads did appear to have traces of *iso*xanthopterin on subjective inspection of the chromatograms, so probably not all of the treated tubes were totally without effect. The difference between treated and untreated tubes is

TABLE 3—THE ACTION OF INJECTED WILDTYPE MALPIGHIAN TUBES WITH AND WITHOUT ULTRA-VIOLET IRRADIATION ON THE PTERIDINE CONTENT OF ry^2-FLIES. DATA ARE EXPRESSED IN TERMS OF ARBITRARY UNITS OF FLUORESCENCE PER HEAD

	+-control		ry^2 -control		ry^2 with irradiated +-tubes		ry^2 with untreated +-tubes	
	n	Mean	n	Mean	n	Mean	n	Mean
Drosopterines	26	32·4 ± 1·20	19	14·1 ± 0·83	17	15·8 ± 0·92	14	26·1 ± 1·33
*Iso*xanthopterin	26	20·2 ± 0·65	19	10·8 ± 0·66	17	11·7 ± 0·74	14	14·8 ± 0·64

highly significant ($p < 0{\cdot}01$). A reasonable explanation, although certainly not the only one, would appear to be that transplanted tissues must produce some substance in the host rather than simply acting as a vehicle for the transport of this substance from wildtype to ry^2.

Attempts to phenocopy ry^2 through treatment with 2-amino-4-hydroxypteridine-6-aldehyde were unsuccessful. This substance is well known as an extremely potent inhibitor of xanthine oxidase (see DE RENZO, 1956). It also inhibits preparations of *Drosophila* xanthine dehydrogenase (private communication of Prof. H. K. MITCHELL). The compound was administered through feeding and injection of suspensions. It appeared to be converted in part to a compound which did not differ chromatographically from 2-amino-4-hydroxypteridine-6-carboxylic acid. Wildtype Malpighian tubes which had been treated for 1 hr with a suspension of the inhibitor were less effective than controls in altering the phenotype of ry^2 hosts although the inhibition was by no means complete. However, the treatment was clearly somewhat toxic and the results can readily be accounted for by the toxicity of the treatment. The apparently direct relationship between the *ry* locus and the enzyme xanthine dehydrogenase would appear to offer unique opportunities for phenocopy experiments if the enzyme could be effectively inhibited *in vivo*.

REFERENCES

DeRenzo E. C. (1956) Chemistry and biochemistry of xanthine oxidase. *Advanc. Enzymol.* **17**, 293–318.

Forrest H. S., Glassman E., and Mitchell H. K. (1956) Conversion of 2-amino-4-hydroxypteridine to *iso*xanthopterin in *D. melanogaster*. *Science* **124**, 725–726.

Forrest H. S. and Mitchell H. K. (1955) Pteridines from *Drosophila*. III. Isolation and identification of three more pteridines. *J. Amer. chem. Soc.* **77**, 4865–4869.

Glassman E. and Mitchell H. K. (1958) *Genetics*. In press.

Hadorn E. (1956) Erbkonstitution und Merkmalsbildung. *Verh. schweiz. naturf. Ges. (Basel)* **6**, 52–66.

Hadorn E. und Graf G. E. (1958) Weitere Untersuchungen über den nicht-autonomen Pterinstoffwechsel der Mutante *rosy* von *Drosophila melanogaster*. *Zool. Anz.* **160**, 231–243.

Hadorn E., Graf G. E., und Ursprung H. (1958) Der *Iso*xanthopterin-Gehalt transplantierter Hoden von *Drosophila melanogaster* als nicht-autonomes Merkmal. *Rev. suisse Zool.* **65**, 335–342.

Hadorn E. und Kühn A. (1953) Chromatographische und fluorometrische Untersuchungen zur biochemischen Polyphänie von Augenfarb-Genen bei *Ephestia kühniella*. *Z. Naturf.* **10b**, 582–589.

Hadorn E. and Mitchell H. K. (1951) Properties of mutants of *Drosophila melanogaster* and changes during development as revealed by paper chromatography. *Proc. nat. Acad. Sci., Wash.* **37**, 650–665.

Hadorn E. und Schwinck I. (1956) Fehlen von *Iso*xanthopterin und Nicht-Autonomie in der Bildung der roten Augenpigmente bei einer Mutante (*rosy*^{a}) von *Drosophila melanogaster*. *Z. indukt. Abstamm.-u. VererbLehre* **87**, 528–553.

Ursprung H., Graf G. E., und Anders G. (1958) Experimentell ausgelöste Bildung von rotem Pigment in den Malpighischen Gefässen von *Drosophila melanogaster*. *Rev. suisse Zool.* **65**, 449–460.

Viscontini M., Hadorn E., und Karrer P. (1957) Fluorescierende Stoffe aus *Drosophila melanogaster*. 5. Mitteilung. *Helv. chim. acta* **40**, 579–583.

Ziegler-Günder I. und Hadorn E. (1958) Manifestation rezessiver Augenfarb-Gene im Pterin-Inventar heterozygoter Genotypen von *Drosophila melanogaster*. *Z. indukt. Abstamm.-u. VererbLehre* **89**, 221–234.

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 125 *to* 126. *Pergamon Press Ltd., London. Printed in Great Britain*

A SIMPLE ACTIVITY RECORDER

R. H. J. BROWN

Department of Zoology, University of Cambridge

(*Received* 9 *August* 1958)

Abstract—A simple activity recorder has been constructed using transistor circuits and working on infra-red light. It is suitable for long periods of observation, and the recording system can be set up at a distance from the animals.

VOL. 3 1959

THE development of this apparatus arose from the need to record the activity phases of insects over long periods of time with a device which would disturb the animals as little as possible. In particular it was required that all the apparatus apart from the detecting device itself should be kept at a distance from the animals, so that it could be tested, recording paper changed, and so on, without affecting them.

Since it is clear that insects are generally not sensitive to the red end of the spectrum (WIGGLESWORTH, 1953), it is possible to detect activity by a photoelectric system using red or infra-red light.

FIG. 1. Layout of the detecting system. On the right is the lamp and lens mounting, on the left the photocell unit. These two can be separated by any convenient distance up to several feet.

The apparatus is divisible into two sections, the detection system shown in Fig. 1, and the recording circuit shown in Fig. 2. The detection system takes the form of two small stands each carrying a tube with a short focus lens of about 1 cm diameter. In one tube, at the focus, there is a 6 V 0·3 A torch bulb; the other tube carries a phototransistor type OCP71, which has a high red sensitivity. These small stands can be placed on either side of the region to be observed, and adjusted so that the light from the lamp is directed onto the photocell. The change in photocell current due to total or partial interruption of the light beam is used to operate the recording circuit shown in Fig. 2.

When steady light is falling on the OCP71 the current is constant, but when the light is obscured the current falls. The resultant current flow into the 50 μF condenser causes the OC71 transistor current to rise and operate the relay. The CV448 germanium diode prevents the condenser from developing a reverse charge when the light increases and thereby increases the speed of operation of the circuit. The 2 mA meter is required when setting up the apparatus; it enables the lamp and photocell to be correctly aligned, since the reading is maximal when the two are exactly in line with each other.

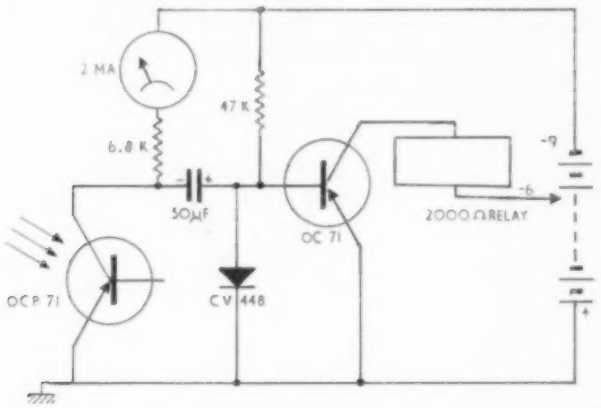


Fig. 2. The circuit of the photocell amplifier. This includes the primary relay, but not the recording system.

The red light is obtained by running the lamp far below its rated voltage. This is achieved by a variable series resistance, which is increased until the meter current is reduced to a suitable value. Since the amplifier needs a certain change of photocell current in order to operate the relay, it will be obvious that the actual steady current required for reliable operation will depend, in a particular case, on a number of factors—the size of the animal, for example, will determine the fraction of the beam interrupted. Generally the steady current is set at about 0·5 mA. This corresponds to about 0·5 V on the lamp, which gives only a dim red glow from the centre of the filament.

The recording system can be of several types; the relay at present being used to operate a simple marker on a smoked drum. Clearly many different systems are available.

Two such units have been in continuous use for over six months recording the rhythmic activity of cockroaches. They differ from the foregoing description only in that the power supply is derived from the A.C. mains by a transformer and rectifiers; an addition which is convenient where the apparatus is not required to be portable and self-contained.

REFERENCE

Wigglesworth V. B. (1953) *The Principles of Insect Physiology*. Methuen, London.

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 127 *to* 131. *Pergamon Press Ltd., London. Printed in Great Britain*

THE INFLUENCE OF DIETARY AMINO ACIDS ON THE BLOOD AMINO ACIDS OF THE GERMAN COCKROACH, *BLATTELLA GERMANICA* (L.)*

JACQUES L. AUCLAIR†

Department of Entomology, Cornell University, Ithaca, N.Y.

(*Received* 22 *August* 1958)

Abstract—Amino acids were fed to the German cockroach, *Blattella germanica* (L.), and a semi-quantitative analysis of the free amino compounds of the blood of the roach was made by paper chromatography. The results indicate that certain dietary amino acids may influence the quality and concentration of amino acids in the blood. α-Amino-butyric acid, hydroxyproline, phenylalanine, and taurine normally absent in the blood may be detected in it if given in the diet. DL-Homocystine is apparently not absorbed through the gut. DL-Methionine can give rise in the blood to α-amino-butyric acid and can increase the blood content in lysine and histidine. The metabolism of D-glutamic acid is different from that of L-glutamic acid. Feeding experiments involving L-cystine, β-alanine, and DL-serine suggest that the metabolism of these three compounds is interrelated. When L-glutamic acid, L-glutamine, α-ketoglutaric acid, DL-alanine, DL-aspartic acid, and L-asparagine were fed respectively, the results from the blood analyses suggested the existence in the roach of a deamination and transamination scheme similar to that demonstrated in mammals.

VOL.
3
1959

INTRODUCTION

THE importance of amino acids in insect nutrition is well established and several amino acids are essential for the normal growth of insects (e.g. ALBRITTON, 1954; LIPKE and FRAENKEL, 1956). Furthermore, since the review paper by RAPP (1947) on insect haemolymph, a large variety of free amino acids were identified as normal constituents of insect blood (e.g. PRATT, 1950; ROEDER, 1953; AUCLAIR and DUBREUIL, 1953), but the intermediary metabolism of these compounds is still little known. The analysis of the free amino acids of the blood of the German cockroach, *Blattella germanica* (L.) made by PRATT (1950) indicated that alanine, arginine, asparagine, aspartic acid, β-alanine, cystine, glutamic acid, glutamine, glycine, leucine and/or *iso*leucine, lysine, methionine, proline, serine, threonine, tyrosine, and valine were normal constituents. This is a report on the influence of dietary amino acids on the blood amino acids of *B. germanica* (L.).

MATERIALS AND METHODS

The German roach was reared as described in an earlier paper (AUCLAIR, 1959), and groups of about 75 adults were placed in battery jars. They were

* This investigation was aided by a grant from the Lalor Foundation, Wilmington, Delaware. The material presented herewith was part of a doctorate thesis submitted to the Graduate School, Cornell University, 1949.

† Entomologist, Science Service Laboratory, St. Jean, Qué., Canada.

starved during 2–5 days during which time they were given water only. The experimental groups were then fed a 2 : 1 mixture of dextrin and one amino acid during a period of several days, and the control groups were fed dextrin only. In a few subsequent experiments, the dextrin was replaced by the complete diet (AUCLAIR, 1959) which was given to the experimental groups as a 1 : 1 mixture with the studied amino acid. The control groups were given a 1: 1 mixture of the complete diet and dextrin. Blood samples of 50, 75, and 100 µl were taken from the experimental and control groups at 2-day intervals beginning with the first or the second day of the feeding period. It required the bleeding of from 10 to 30 insects to yield the above amounts of blood, so that individual variations in metabolism were mostly eliminated. Bled insects were discarded and the same group of insects was not used for two different feeding experiments. The blood samples were collected and processed as described by AUCLAIR and DUBREUIL (1953) prior to chromatographic separation. The method of paper chromatography as described by CONSDEN *et al.* (1944) was used for the qualitative and semi-quantitative analysis of amino acids. Two-dimensional chromatograms of blood samples from the experimental and control groups were prepared simultaneously and the relative size and intensity of the spots determined visually. The sensitivity of the ninhydrin reaction on the chromatograms being known (PRATT and AUCLAIR, 1948), the missing compounds were therefore at a lower level than the minimum quantity detectable by the ninhydrin reaction.

RESULTS AND DISCUSSION

The blood of control as well as experimental insects indicated the presence of the amino acids reported by PRATT (1950) and the following variations: The occasional absence of aspartic acid, glutamic acid, cystine, and arginine; slight variations in the intensity of the spots (even between controls) indicating a variation in the blood amino acid concentration between groups. However, the results which were most obvious are condensed in Table 1, and they indicate that certain dietary amino acids may influence the quality and concentration of amino acids in the blood, whereas others seem to have little or no influence. Compounds such as α-amino-butyric acid, hydroxyproline, phenylalanine, and taurine, not recorded by PRATT (1950), may be detected in the blood if they are fed to the insect. They could be used as tracers to study the physiology of the digestive tract and the intermediary metabolism.

The diets containing the hydrochloride amino acids or DL-aspartic acid had a poor palatability, whereas the diets containing sulphur amino acids or DL-serine had a high palatability. Homocystine was eaten greedily and yet analyses revealed no change whatever in the blood picture. The faeces of homocystine-dextrin fed insects were white like the diet and gave a very strong ninhydrin reaction. This suggests that homocystine is not absorbed into the blood. Homocystine has apparently not been recorded as yet from insect blood (ROEDER, 1953). The varying increases in blood content in the other compounds fed may be due to differences in palatability, in rate of absorption, of conversion and utilization in

intermediary metabolism, or of excretion. The blood increases in amino compounds were usually more pronounced in the first analysis than in the subsequent ones. This may be explained by the influence of the starvation period on the insect's appetite prior to experimental feeding.

TABLE 1—CHANGES IN THE FREE AMINO COMPOUNDS OF THE BLOOD OF *B. GERMANICA* FED INDIVIDUAL AMINO COMPOUNDS

Amino compound fed	Palatability †	No. of Analyses	Changes in the blood free amino compounds	
			Relative increase in compound fed	Slight increase (incr.) or decrease (decr.) in other amino compounds
DL-Alanine	fair	3	+ + +	glutamine incr. (1)‡
β-Alanine	low	3	+ + +	serine decr. (2)
DL-α-Amino-butyric acid*	fair	2	+ + +	
L-Arginine HCl	poor	2	—	lysine incr. (1)
L-Asparagine	fair	2	+	glutamine incr. (2) aspartic incr. (1)
DL-Aspartic acid	poor	5	+	
L-Cysteine HCl	poor	3	—	
L-Cystine	high	11	—	beta-alanine incr. (3) serine decr. (1) disappear (1)
L-Cystine + DL-serine	high	2	+ + + (ser.)	beta-alanine incr. (1)
D-Glutamic acid	fair	2	+	
L-Glutamic acid	fair	2	—	glutamine incr. (2) alanine incr. (1)
L-Glutamine	fair	2	+	proline incr. (1)
Glycine	fair	2	+	
DL-Homocystine*	high	2	—	
L-Hydroxyproline*	fair	2	+	proline decr. (2)
α-Ketoglutaric	low	2	no test	glutamic acid incr. (1)
L-Leucine	fair	2	+	
L-Lysine HC1	poor	2	—	
DL-Methionine	fair	6	+ + +	lysine incr. (3) histidine incr. (2) α-amino-butyric incr. (2)
DL-Phenylalanine	fair	2	+ + +	
DL-Serine	high	3	+ + +	
Taurine*	high	3	+ + +	
DL-Threonine	fair	2	+	
L-Tyrosine	fair	2	+	

* Compound absent in normal *B. germanica* blood.

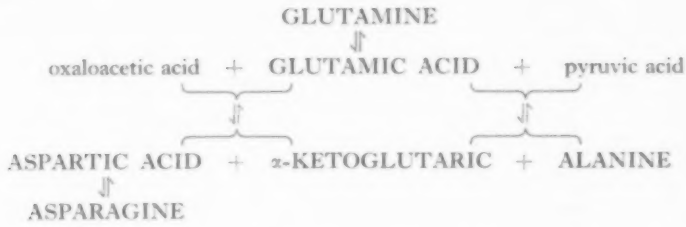
† Poor: little or not eaten—low: little eaten—fair: moderately eaten—high: greedily eaten.

‡ The numbers in parentheses indicate the number of analyses in which the results were observed.

Dietary D-glutamic acid produced a small increase of glutamic acid in the blood, but dietary L-glutamic acid produced an increase in glutamine and alanine. This indicates once more the physiological significance of the spatial configuration of the amino group in the molecule.

VOL. 3 1959

Dietary alanine and α-ketoglutaric acid produced an increase in blood glutamine and glutamic acid respectively. Dietary L-glutamic acid produced an increase in blood glutamine and alanine. Dietary asparagine produced an increase in blood aspartic acid and glutamine. The relationships of these compounds in insects are probably identical to those reported in some plant and animal tissues (BRAUNSTEIN, 1947; BALDWIN, 1957) where transaminases have been shown to catalyse the reactions:



The compounds printed in capital letters were fed to the insects. The reversible reaction, aspartic acid = asparagine, has not been demonstrated in mammals but is indicated in insects by the fact that asparagine is readily synthesized in the German roach (AUCLAIR, 1949) and that ingested asparagine produced an increase in blood aspartic acid (Table 1). Likewise, BHEEMESWAR and SREENIVASAYA (1952) presented evidence for the presence of a transaminase in the haemolymph, intestine, and silk gland of the silkworm, *Bombyx mori* (L.), which catalyses the following reaction: L-aspartic acid + α-ketoglutaric acid ⇌ oxaloacetic acid + L-glutamic acid. KILBY and NEVILLE (1957) working on the desert locust, *Schistocerca gregaria* Forsk., reported that homogenates of fat body catalyse transamination reactions between α-ketoglutarate and several amino acids, and that fat body, Malpighian tubes, and mid-gut wall homogenates are able to convert glutamic acid into glutamine.

Dietary cystine produced an increase in β-alanine and a decrease in serine, β-alanine produced a decrease in serine, and serine did not produce an increase in the above two compounds. These results suggest that the metabolism of cystine, serine, and β-alanine is interrelated. This is to be expected from their molecular structure.

Methionine can be metabolized to α-amino-butyric acid in the German roach (Table 1), again showing a similarity with the findings in mammals (DENT, 1947). Where α-amino-butyric acid is absent from the diet but occurs naturally in insect blood (e.g. in three insect species, PRATT, 1950), it may be assumed that its main source is methionine. Methionine also has an influence on the lysine and histidine content of the blood (Table 1).

Acknowledgements—The writer wishes to express his sincere gratitude and appreciation to the project leader, Dr. R. L. PATTON, professor of insect physiology at Cornell University, for his encouragement and assistance throughout this work and to the Lalor Foundation of Wilmington, Delaware, for financial support.

REFERENCES

ALBRITTON E. C. (1955) *Standard values in Nutrition and Metabolism.* W. B. Saunders Co., Philadelphia.

AUCLAIR J. L. (1949) *Qualitative studies on the Metabolism of Amino Acids in Insects.* Doctorate thesis, Cornell University, Ithaca, N.Y.

AUCLAIR J. L. (1959) Amino acid oxidase activity in the fat body and Malpighian tubes of some insects. *J. Ins. Physiol.* **3**, 57–62.

AUCLAIR J. L. and DUBREUIL R. (1953) Etude sur les acides aminés libres de l'hémolymphe des insectes par la méthode de chromatographie sur papier filtre. *Canad. J. Zool.* **31**, 30–41.

BALDWIN E. (1957) *Dynamic Aspects of Biochemistry.* Cambridge Univ. Press, London.

BHEEMESWAR B. and SREENIVASAYA M. (1952) Occurrence of transaminase in the silkworm, *Bombyx mori* L. *Current Sci.* **21**, 253–255.

BRAUNSTEIN A. E. (1947) Transamination and the integrative functions of the dicarboxylic acids in nitrogen metabolism. *Adv. Prot. Chem.* **3**, 2–52.

CONSDEN R., GORDON A. H., and MARTIN A. J. P. (1944) Qualitative analysis of proteins: a partition chromatographic method using paper. *Bioch. J.* **38**, 224–262.

DENT C. E. (1947) Methionine metabolism and α-amino-n-butyric acid. *Science* **105**, 335.

KILBY B. A. and NEVILLE E. (1957) Amino acid metabolism in locust tissues. *J. exp. Biol.* **34**, 276–289.

LIPKE H. and FRAENKEL G. (1956) Insect nutrition. *Ann. Rev. Ent.* **1**, 17–44.

PRATT J. J. Jr. (1950) A qualitative analysis of the free amino acids in insect blood. *Ann. Ent. Soc. Amer.* **43**, 573–580.

PRATT, J. J. Jr. and AUCLAIR J. L. (1948) The sensitivity of the ninhydrin reaction in paper partition chromatography. *Science* **108**, 213.

RAPP Janet L. (1947) Insect hemolymph: a review. *J. New York Ent. Soc.* **55**, 295–308.

ROEDER K. D. (1953) *Insect Physiology.* John Wiley, New York.

VOL.
3
1959

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 132 *to* 145. *Pergamon Press Ltd., London. Printed in Great Britain*

PIGMENT COMPOSITION IN THE PUPAL CUTICLES OF TWO COLOUR TYPES OF THE SWALLOWTAILS, *PAPILIO XUTHUS* L. AND *P. PROTENOR DEMETRIUS* CRAMER

EIJI OHNISHI*

Department of Biology, Faculty of Science Tokyo Metropolitan University, Setagayaku, Tokyo, Japan

(*Received* 25 *August* 1958)

Abstract—In the swallowtails of the genus *Papilio*, there are two pupal types, green and brown. These types are determined just before pupation by environmental stimuli (OHNISHI and HIDAKA, 1955). The pigment composition of the pupal cuticles of these types was analysed. In the green pupae three carotenoids, β-carotene, lutein, and an unknown carotenoid were found. Neither β-carotene nor lutein was detected in the brown pupae, but an astaxanthin-like carotenoid and a group of previously unknown carotenoids, which were called "papilioerythrins", were found. A blue bile pigment, *meso*biliverdin, was extracted from both types of pupae. Its content in the brown pupae was 66 per cent (*P. xuthus*) or 34 per cent (*P. protenor demetrius*) of that in the green pupae. Three pterins, xantho-, *iso*xantho-, and leucopterin, were detected in the cuticles of both types by paper chromatography. These findings are discussed in connexion with a hormone, which is secreted from the prothoracic ganglion and is the immediate factor controlling these pupal types.

INTRODUCTION

IN the pupae of Papilionid butterflies, there are two colour types, green and brown. These two colour types differ distinctively not only in their colouration but also in their morphological aspects, although intermediate types appear in some species such as *P. xuthus* L. This paper deals with the pigment composition of the pupal cuticles of the two colour types in the swallowtails, *Papilio xuthus* L. and *P. protenor demetrius* Cramer.

It has long been known that some butterflies possess the ability of adjusting their pupal colouration in response to environmental stimuli. This phenomenon has mostly been studied in *Pieris*, *Vanessa*, and *Papilio*. In *Pieris*, the colour types of the pupa seem to be determined by the colour of the light received by the eyes of the pupating larva, and some authors claimed that yellow, green, and especially orange light induce the production of green pupae; whereas other coloured light, such as red, brown, or purple causes greyish pupae (*cf.* WIGGLESWORTH, 1953).

In the swallowtails belonging to the genus *Papilio* WOOD (1867) and BARBER (1874) described the change of pupal colouration in response to the colour of the sites where they pupate. But a more recent study (SEVASTOPULO, 1947) failed to

* Present address: Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California, U.S.A.

show any relationship between the types of the pupae and the colour of the containers where they were forced to pupate.

In 1955, using *P. xuthus* and *P. protenor demetrius*, OHNISHI and HIDAKA were able to show that pupae which have pupated on green twigs of the food plant are always of the green type, while those pupating on dead branches are of the brown type. Further, it was shown that the same relation also holds even in darkness, indicating that visual perception is not so important for the determination of the pupal types as has hitherto been believed. Instead, some unknown sensory system is concerned in the mediation of the environmental stimuli. HIDAKA (1956) suggested that the olfactory sense is responsible. ISHIZAKI and KATO (1956) reported that low temperature, darkness during the larval life, and low humidity at the time of pupation increased the production of orange type pupae in *Papilio xuthus*.

Analysis of this phenomenon from the physiological viewpoint has been made by HIDAKA (1956), who was able to show that hormonal control was involved in the determination of pupal types. HIDAKA showed by his ligation and transplantation experiments that the environmental stimuli, which are received by some receptors and mediated by the nervous system, cause the secretion from the prothoracic ganglion of a hormone, which is the immediate factor in the determination of pupal types.

VOL. 3 1959

As a first step towards the understanding of the phenomenon at a biochemical level, the present author analysed the pigment composition of the pupal cuticles of both pupal types, using *P. xuthus* and *P. protenor demetrius* as materials. Although the small amount of material did not allow a detailed analysis of the individual pigments, the results so far obtained are nevertheless of interest.

The pigmentary system of the green pupae is a typical "insectoverdin" type, which is common among green insects such as locusts or mantis (JUNGE, 1941; GOODWIN, 1952). The term "insectoverdin" was introduced by JUNGE to designate the green pigment complex that consists of blue and yellow chromoproteins, the prosthetic group of the blue protein being a bile pigment and that of the yellow one carotenoid(s). The blue chromoprotein, a globulin, was extracted from the cuticles of the green pupae, and the prosthetic group, separated from the protein by acid treatment, was analysed. Judging from its chemical and spectrophotometrical nature, it seemed to be identical to *meso*biliverdin, which is always encountered in the insectoverdin complex (GOODWIN, 1952).

The carotenoids, extracted from the green pupal cuticles of the two species, could be differentiated into two major and one minor components by chromatography. The two major carotenoids were identified as β-carotene and lutein (xanthophyll) by their absorption spectra in various solvents and other chemical properties. Major pigments responsible for the colouration of the brown pupae are a bile pigment, carotenoids, and melanin or quinone-tanned protein. Neither lutein nor β-carotene could be extracted from the cuticles of the brown pupae. Instead, an astaxanthin-like carotenoid and a group of previously unknown carotenoids, tentatively called "papilioerythrins" in this paper, were obtained from them.

In view of the fact that the two pupal types are determined by the environmental factors shortly before the pupation and are genetically identical, it is quite interesting to find entirely different groups of carotenoids in the cuticles of the two pupal types. Three pterins were found in both pupal types, although their contribution to the pupal colouration seems to be negligible. They are considered to be *iso*xantho-, leuco-, and xanthopterin from their R_f values on paper chromatography.

MATERIALS AND METHODS

Materials

Eggs and larvae were collected from the field and reared on food plants, which were mostly the leaves of "Karatachi", *Poncirus trifoliata* L. Pupae were dissected and cuticles were separated from other tissues by scraping and washing with running water. The cuticles were then dried in the air and stored in a desiccator.

Methods

(a) *Extraction and chromatography of carotenoids*

The dried cuticles were crushed and ground to powder. The powder was moistened with a few drops of diluted acetic acid and extracted repeatedly with acetone. The acetone extract was concentrated under reduced pressure in a stream of dried nitrogen. The concentrated extract was added to a small amount of light petroleum ether and washed with water. The extract was dried with anhydrous sodium sulphate and chromatographed on alumina (product of Wako Pure Chemicals, specially prepared for chromatography). Sometimes alumina, deactivated by methyl alcohol (GOODWIN and SRISUKH, 1949), was used as the adsorbent of the chromatogram. Each band on the chromatogram was cut out and eluted with petroleum ether containing either acetone or ethyl alcohol. Absorption spectra of the isolated pigments were measured by a Hitachi photoelectric spectrophotometer. All solvents used in these experiments were purified by distillation.

(b) *Extraction and isolation of bile pigment*

(1) *Extraction of the blue chromoprotein.* Powdered cuticles were extracted with N/10 sodium bicarbonate, and the extract was mixed with ammonium sulphate to $\frac{1}{10}$ saturation to remove some inert protein. The concentration of ammonium sulphate was then increased to $\frac{1}{3}$ saturation, which is sufficient to precipitate the chromoprotein. The precipitate was dissolved in water and the precipitation by ammonium sulphate was repeated once more.

(2) *Isolation of the prosthetic group, the bile pigment.* When the aqueous solution of the chromoprotein was acidified with acetic acid, the prosthetic group was detached from the protein and could be extracted with diethyl ether. The ether extract was washed with water and concentrated *in vacuo*. The residue was dissolved in methyl alcohol or ethyl acetate. The pigment was further purified either by adsorbing onto acid washed talc and eluting it with acidic alcohol, or by washing the ethyl acetate solution with N/10 NaOH and water.

(c) *Separation and identification of pterins by paper chromatography*

Pterins were extracted with N/10 sodium bicarbonate from the powdered cuticles. After precipitating the chromoprotein by the addition of ammonium sulphate to $\frac{1}{3}$ saturation, solid ammonium sulphate was further added to saturation and the precipitated proteins were centrifuged off. The supernatant was extracted with liquid phenol, and pterins were further transferred to the aqueous layer by the addition of diethyl ether (CRAMMER, 1948). The aqueous extract was applied to paper chromatography. Five per cent sodium citrate or a mixture of *n*-butanol, acetic acid, and water were employed as the developer. Synthetic *iso*xantho-, leuco-, and xanthopterin (kindly supplied by Prof. M. AKINO) were applied simultaneously and their R_f values and the colour of their fluorescence were compared.

RESULTS

(I) *Carotenoids*

(a) *Carotenoids in the cuticles of the green pupae.* The most remarkable difference in the pigment composition between the two pupal types is that found in the composition of carotenoids. As shown in Fig. 1, the carotenoids of the green

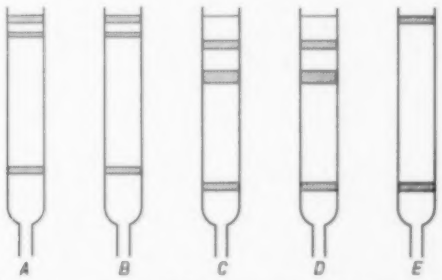


FIG. 1. Schematic representation of chromatograms of carotenoids obtained from pupal cuticles of *Papilio* and from the lobster, *Panulirus japonicus*. (A) Carotenoids obtained from the green pupae of *P. xuthus* L. Bands are those of lutein, unknown carotenoid, and β-carotene (from top to bottom). (B) Carotenoids extracted from the green pupae of *P. protenor demetrius* Cramer. Carotenoids adsorbed are the same as those of *P. xuthus* green pupae. (C) Carotenoids obtained from the brown pupae of *P. xuthus* L. Bands are those of px–1, px–2, and are astaxanthin-like carotenoid. (D) Carotenoids obtained from the brown pupae of *P. protenor demetrius* Cr. Bands are those of pp–1, pp–2, and an astaxanthin-like carotenoid. (E) Carotenoids obtained from the carapace and hypodermis of the lobster, *Panulirus japonicus*. Bands are those of astaxanthin and an astaxanthin ester. Adsorbent: alumina (column A, B), deactivated alumina (column C, D, E). Developer: petroleum ether (A, B), petroleum ether containing 0·5–5 per cent ethyl alcohol (C, D, E).

pupae were separated into three bands on the chromatogram; yellow band on the top, orange-yellow band moving fast, and faint pink band below the yellow band. No remarkable difference in the pattern of the chromatogram was found between the green types of the two species employed. The orange-coloured carotenoid,

VOL. 3 1959

which was adsorbed at the lower part of the chromatogram, seems to be a hydrocarbon carotenoid from its epiphasic nature on partition between petroleum ether and 90 per cent methyl alcohol and its behaviour on chromatograms. The absorption spectrum of the pigment resembled closely that of β-carotene. Table I shows a comparison of the absorption maxima between the isolated pigment and a commercial sample of β-carotene (product of Dr. Th. Schuchardt, Munich).

TABLE 1—ABSORPTION MAXIMA OF THE ISOLATED PIGMENT AND THOSE OF β-CAROTENE

Solvent / Pigment	Petroleum ether (b.p. 40–60°C)	CS_2
Pigment from *P. protenor demetrius*	(426) 450 480	485 514
β-carotene (product of Th. Schuchardt)	(428) 452 481	
β-carotene (according to GOODWIN, 1952)	451 484 (450)*(478)*	485 521

* Numbers in parentheses are the values obtained using hexane as the solvent.

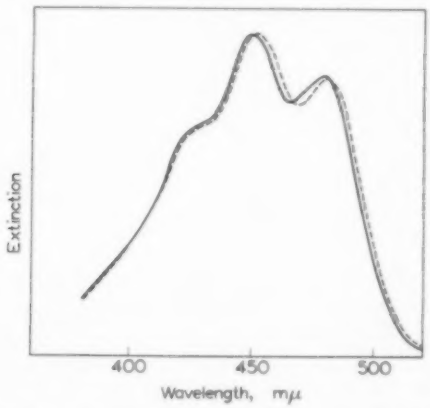


FIG. 2. The absorption spectrum of the carotenoid extracted from the green pupae of *P. protenor demetrius* Cr. and supposed to be β-carotene (solid line), and that of β-carotene, the product of Schuchardt (broken line). Solvent is petroleum ether (b.p. 40–60°C).

Slight displacement of the pupal pigment towards shorter wave-lengths is probably due to the contamination of isomers, such as neo-β-carotene B, which might be produced during the procedure of isolation. Identity of the pupal pigment with β-carotene was confirmed by mixed chromatography. Mixture of the two pigment samples, the pupal pigment and the commercial sample, did not separate after chromatographing through a long column (20 cm), using petroleum ether

containing 0·5 per cent (v/v) acetone as the developer. The yellow carotenoid, which was adsorbed on the top of the column, was hypophasic on partition between petroleum ether and 90 per cent methyl alcohol. The absorption spectrum resembled closely that of lutein (Fig. 3).

TABLE 2—ABSORPTION MAXIMA OF THE ISOLATED PIGMENT AND THOSE OF LUTEIN

Solvent / Pigment	Petroleum ether (b.p. 40–60°C)		CS_2		
Pigment from *P. protenor demetrius*	442	472	445	477	509
Lutein (according to KARRER and JUCKER, 1950)	447·5	477·5	445	477	508

TABLE 3—RELATIVE ABUNDANCE OF β-CAROTENE AND LUTEIN IN THE CUTICLES OF THE TWO SPECIES

Species / Carotenoid	*P. xuthus*	*P. protenor demetrius*
β-carotene	56 per cent	34 per cent
Lutein	44 per cent	66 per cent

The positions of the absorption maxima in two solvents are presented in Table 2, together with the value of lutein described in the literature. The positions of the maxima in petroleum ether differed slightly from those described in the literature, presumably owing to the difference in the composition of petroleum ether, as those obtained in carbon disulphide approximated closely to those described in the literature. The third component, which was adsorbed just below the lutein band on the chromatogram, was epiphasic on partition between petroleum ether and 90 per cent methyl alcohol. The absorption spectrum of the isolated pigment somewhat resembles that of astaxanthin as it is bell-shaped and had a single maximum (Fig. 3, dotted line), but the position of the maximum is different from it (maximum of the pupal pigment: 454 mμ; astaxanthin ester from the lobster, *Panulirus japonicus*, 464 mμ in petroleum ether). Relative abundance, roughly estimated by their optical density at maxima, of β-carotene and lutein in the cuticles of the two species is presented in Table 3. As the quantity of the third component was very slight, it is neglected here.

(b) *Carotenoids in the cuticles of the brown pupae.* The pigments of the brown pupae were adsorbed on the top of the chromatographic column and did not move when petroleum ether only was used as the developer (adsorbent: deactivated alumina). However, the carotenoids separated into three components when a

VOL. 3 1959

small amount of acetone or ethyl alcohol was mixed with petroleum ether of the developer.

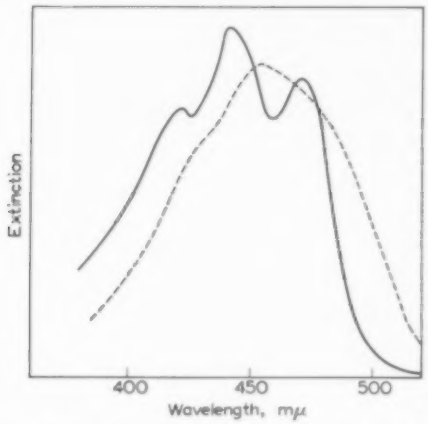


FIG. 3. The absorption spectrum of the carotenoid obtained from the green pupae of *P. xuthus* and supposed to be lutein (solid line), and that of the third component obtained from the green pupae of *P. protenor demetrius* (broken line), in petroleum ether (b.p. 40–60°C).

Although the patterns of the chromatograms were quite similar in the two species, the nature of the carotenoids eluted from the column was slightly different

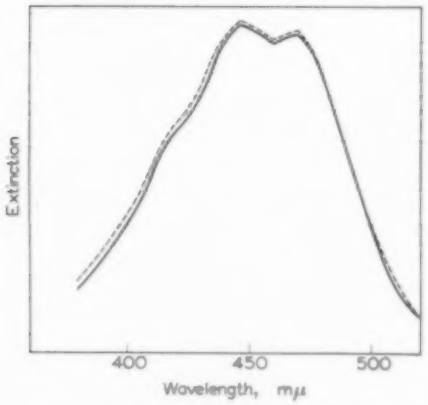


FIG. 4. The absorption spectra of papilioerythrins px–1 (broken line) and px–2 (solid line) obtained from the brown pupae of *P. xuthus* in petroleum ether (b.p. 40–60°C).

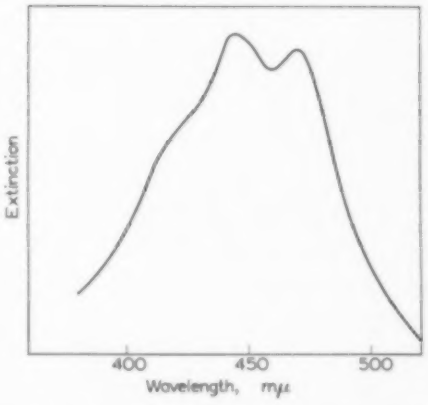


FIG. 5. The absorption spectrum of papilioerythrin pp–2 obtained from the brown pupae of *P. protenor demetrius*, in petroleum ether (b.p. 40–60°C).

between the species in respect to their absorption spectra and their nature on partition between petroleum ether and 90 per cent methyl alcohol. The two

carotenoids eluted from the upper two bands have quite similar absorption spectra in both species. This fact seems to indicate that they have closely related chemical constitutions. Although there is a slight difference between the species, the absorption curves of these carotenoids are unique (Fig. 4 and 5). This group of pigments

TABLE 4—ABSORPTION MAXIMA OF PAPILIOERYTHRIN × −2

Solvent	Absorption maxima		
Petroleum ether (b.p. 40–60°C)	(415–20)	446	468
CS_2	(445–50)	478	502
Benzene	(435)	462	484

is tentatively called "papilioerythrins" in this paper. Papilioerythrins extracted from the cuticles of *P. xuthus* are called papilioerythrin x-1, papilioerythrin x-2 (abbreviated as px-1 and px-2) and those obtained from *P. protenor demetrius* are called pp-1, pp-2. The numbers given after the hyphen express the order on the chromatogram (from top to bottom). The pigment px-1 and px-2 were both hypophasic on partition between petroleum ether and 90 per cent methyl alcohol. Their absorption spectra showed no significant difference (Fig. 4), and the positions

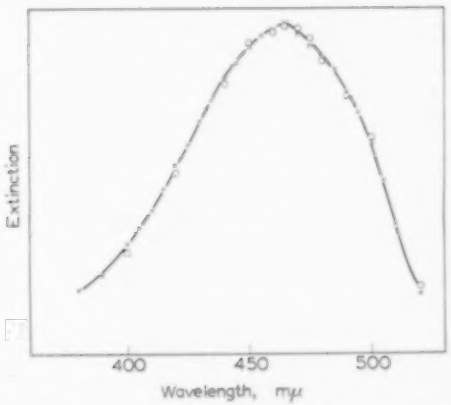


FIG. 6. The absorption spectrum of astaxanthin-like carotenoid, obtained from the brown pupae of *P. xuthus* and that of astaxanthin ester obtained from the lobster, *Panulirus japonicus*. The values of extinction of the pupal pigment are indicated by the mark (x), and the values of astaxanthin ester of the lobster are shown by the mark (o). The scales of both carotenoids are adjusted to make values of E_{max} equal. The solvent is petroleum ether (b.p. 40–60°C).

of the absorption maxima are presented in Table 4. The pigment pp-1 was epiphasic on partition between petroleum ether and 90 per cent methyl alcohol. but pp-2 was hypophasic. As the quantity of pp-1 was slight, it was not feasible to trace its whole spectral curve, but the positions of the absorption maxima are

the same as those of pp-2. The maxima of the curve, shown in Fig. 5, are at 444 mμ and at 470 mμ in petroleum ether (b.p. 40–60°C).

An astaxanthin-like carotenoid, which was extracted from *P. xuthus* cuticles and adsorbed at the lowest part of the column, was epiphasic on partition between petroleum ether and 90 per cent methyl alcohol. The absorption spectrum of this pigment overlapped completely with that of astaxanthin ester, which was obtained

TABLE 5—RELATIVE ABUNDANCE OF CAROTENOIDS IN THE CUTICLES OF THE BROWN TYPE PUPAE

P. xuthus brown type	*P. protenor demetrius* brown type
Papilioerythrin x-1 5·3 per cent	Papilioerythrin p-1 14·5 per cent
Papilioerythrin x-2 63·5	Papilioerythrin p-2 40·0
Astaxanthin-like 31·2	Astaxanthin-like 45·5

in a crystalline form from the carapace and hypodermis of the Japanese lobster, *Panulirus japonicus* (Fig. 6). The astaxanthin-like carotenoid, obtained from the cuticles of *P. protenor demetrius*, was epiphasic on partition between petroleum ether and 90 per cent methyl alcohol. The absorption curve also resembled that of astaxanthin, but the shape of the top was broader than that of astaxanthin (Fig.

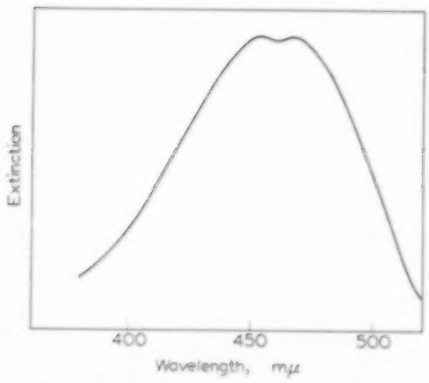


FIG. 7. The absorption spectrum of astaxanthin-like carotenoid obtained from the brown pupae of *P. protenor demetrius* in petroleum ether (b.p. 40–60°C).

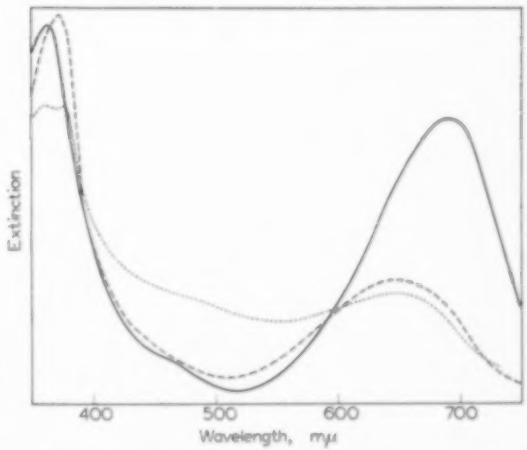


FIG. 8. The absorption spectrum of the blue chromoprotein in phosphate buffer (*p*H 7·0, dotted line) and absorption spectra of the bile pigment in methyl alcohol (broken line) and in methyl alcohol containing 5% HCl (solid line).

7). The position of the maximum in petroleum ether was 455–465 mμ. Relative abundance of these pigments in the cuticles of both species is shown in Table 5. Rough estimates were made by comparing their optical density at their maxima.

(II) *Blue bile pigment*

The absorption spectrum of an aqueous solution of the chromoprotein is shown in Fig. 8 (dotted line). The prosthetic group, a bile pigment, could be readily detached from the protein by making the solution acidic. The pigment was extracted with diethyl ether. The pigment could be transferred from diethyl ether to 1 N NCl. The isolated pigment was positive in the Gmelin test and negative in the diazo reaction. The absorption spectra in various solvents besides those described above, indicate that the pigment is a kind of bile pigment of the bilatriene type (LEMBERG and LEGGE, 1949). The positions of maxima in some solvents are compared with those of *meso*biliverdin in Table 6.

TABLE 6—ABSORPTION MAXIMA OF THE ISOLATED BILE PIGMENT AND THOSE OF *meso*BILIVERDIN

Pigment	Absorption maxima in					
	Methyl alcohol		Methyl alcohol containing 5% HCl		Ether or dioxane	
Pigment from *P. xuthus*	372	650	362	690	373	650
*Meso*biliverdin (LEMBERG and LEGGE, 1949)			363	670		
*Meso*biliverdin (GOODWIN and SRISUKH, 1951)			367	647		636
*Meso*biliverdin (WIELAND and TARTTER, 1940)			364	650		

By the addition of zinc acetate to the alcoholic solution of the pigment, the colour of the solution changed to green, indicating the formation of zinc-pigment complex. The absorption spectrum of the complex had two maxima at 640 mμ and at 685 mμ. The addition of a minute quantity of iodine to the ammoniacal alcoholic solution of the complex changed the colour of the solution to sky blue, and the solution emitted a strong red fluorescence. These are indications that the zinc complex was changed to biliviolinoid type (LEMBERG and LEGGE, 1949). The absorption maxima of this biliviolinoid complex was at 623 mμ. All of these characters indicate that the pigment is *meso*biliverdin. The only discrepancy between the isolated pigment and *meso*biliverdin was the stability of the pigment in concentrated sulphuric acid at 100°C. When dissolved in concentrated sulphuric acid, the pigment exhibits a bluish green colour, which changed to green after 10 min boiling, to olive green after 30 min, and to greenish olive after 1 hr at 100°C. According to WIELAND and TARTTER (1940), *meso*biliverdin (pterobilin in their terminology) extracted from the wings of butterflies resisted the same treatment as long as 9 hr. But *meso*biliverdin extracted from other sources was less stable (JUNGE, 1941; GOODWIN and SRISUKH, 1951).

Relative abundance of the pigment between the two pupal types was estimated by the following method: 500 mg of cuticle powder was placed in a small Soxhlet

and extracted for 3 hr using methyl alcohol containing 5 per cent HCl as the extractant. The extract obtained was placed in a refrigerator for one night, and the precipitate produced was centrifuged off. Optical density of the extract at 640 mμ was taken as the measure of the content of the pigment.

In *P. xuthus*, the content of the pigment in the brown pupae was about 66 per cent of that contained in the green pupae. In *P. protenor demetrius* the difference in the content between the types was more remarkable: the content in the cuticles of the brown pupae was only 34 per cent of that found in the cuticles of the green pupae.

(III) *Pterins*

Judging from the behaviour on paper chromatograms and from the colour of their fluorescence, the cuticles of both pupal types seem to contain at least three pterins, xanthopterin, *iso*xanthopterin, and leucopterin. Among these, *iso*xanthopterin was most abundant and xanthopterin least abundant. No recognizable difference was found between the two pupal types in both species.

DISCUSSION

The biochemistry of insect pigments has been studied most extensively in the desert locust, *Schistocerca gregaria* F., and in the African locust, *Locusta migratoria migratorioides* R and F, by GOODWIN and SRISUKH (1948–52, see GOODWIN, 1952). They identified in the integument of locusts carotenoids (β-carotene and astaxanthin), *meso*biliverdin, insectorubin, melanin, pterins, and flavin, and they compared the pigment composition between *solitaria* and *gregaria* phases. Locusts of phase *solitaria* differ from *gregaria* locusts in the following respects: (a) no melanin is present even in hoppers, (b) the insectorubin content is somewhat reduced in *Locusta* and it is almost completely absent in *Schistocerca*, (c) insectoverdin occurs in the haemolymph and integument.

As regards the determination of these phases, many assumptions and hypotheses have been made since Uvarov first proposed his theory in 1921. The main facts, which were proposed by Uvarov and accepted universally, are as follows: The *gregaria* phase hoppers live in dense swarms, whereas hoppers and locusts live in isolation in the *solitaria* phase. This difference ultimately alters their morphological and physiological characters as well as their behaviour. Recently, NICKERSON (1954) and JOLY (1954) found that the phases are, at least partially, controlled by an endocrine system (corpora allata).

There are three common features in the pupal polymorphism of *Papilio* and the phases of *Locusta* or *Schistocerca*. These are as follows:

(a) The types or phases are determined by the environmental stimuli (they are not determined genetically), and the environmental stimuli do not exert their effect directly, as the effect of temperature on metabolism, but are received by some sense organ(s) and mediated by the nervous system.

(b) Some endocrine system is involved.

(c) The major differences in the pigment composition are the content of melanin and that of insectoverdin.

In *Papilio*, the pigment composition of the two pupal types differs not only in the content of melanin and *meso*biliverdin, but also in the nature of the carotenoids. In the cuticle of the green pupae lutein and β-carotene were found. They derived undoubtedly from food plants, since animals cannot synthesize carotenoids *de novo* and these two are found most abundantly in plants (GOODWIN, 1952).

In the cuticle of the brown pupae new carotenoids, papilioerythrins, were found along with astaxanthin-like carotenoids. Although their chemical constitution is not known they are presumably the oxidized product of β-carotene and/or lutein. Oxidation of β-carotene to astaxanthin in the egg of locust was shown by GOODWIN (1949). Therefore, it is reasonable to assume that β-carotene and/or lutein are oxidized to astaxanthin-like carotenoid and also to papilioerythrins in the body of *Papilio* and that the oxidation takes place only in the presence of the hormone of prothoracic ganglion, since HIDAKA (1956) showed that the green type pupa is the only type produced when the hormone is prevented from action by ligation or extirpation. Considering that melanin formation is also an oxidation process, it may also be suggested that the hormone accelerates biological oxidation in general. In this connexion, destruction of the blue chromoprotein by oxidation is also possible, since destruction of the blue pigment in locust haemolymph, perhaps by the indirect action of tyrosinase, was reported by GOODWIN and SRISUKH (1951).

VOL. 3 1959

Some authors have reported an accelerating or suppressing effect of pterins on melanin formation (ISAKA, 1952; AKINO and ISAKA, 1957; POLONOVSKI, BUSNEL, and BARIL, 1950), and others have stressed the significance of pterins in the mechanism of melanin formation *in vivo* (OSHIMA *et al.*, 1956). But insofar as the pupal pigmentation of *Papilio* is concerned, pterins seem to exert no marked effect on melanin formation, since there is no detectable difference in the nature and content of pterins between the two pupal types.

SUMMARY

(1) Pigment composition of the cuticles of two pupal types, the green and the brown type, in the swallowtails *Papilio xuthus* L. and *P. protenor demetrius* Cr. was studied.

(2) The pigment of the green pupae was a typical "insectoverdin" complex, which is a mixture of blue and yellow chromoproteins. The prosthetic group of the blue chromoprotein is a bile pigment and that of the yellow one is a mixture of carotenoids.

(3) The blue bile pigment is considered to be *meso*biliverdin by its chemical and spectrographic properties.

(4) The bile pigment was also extracted from the cuticles of the brown pupae, but its content was 66 per cent (in the case of *P. xuthus*) or 34 per cent (in the case of *P. protenor demetrius*) as compared with that contained in the cuticles of the green pupae.

(5) Carotenoids extracted from the green pupal cuticles were separated into three components by chromatography. One of the two major carotenoids was

identified as β-carotene and the other seemed to be lutein (xanthophyll) by their behaviour on partition between petroleum ether and 90 per cent methyl alcohol, and on chromatogram, as well as their absorption spectra.

(6) Neither β-carotene nor lutein could be extracted from the cuticles of the brown pupae, but an astaxanthin-like carotenoid and a group of previously unknown carotenoids, which are termed "papilioerythrins" in this paper, were obtained. The absorption spectrum of the astaxanthin-like carotenoid, extracted from the cuticles of the brown pupae of *P. xuthus*, was identical with that of astaxanthin ester obtained in a crystalline form from the lobster, *Panulirus japonicus*. An astaxanthin-like carotenoid extracted from the brown pupae of *P. protenor demetrius* has an absorption spectrum which is similar to, but not identical with, that of the astaxanthin ester. Some spectral properties of papilioerythrins are described.

(7) Three pterins, xantho-, *iso*xantho-, and leucopterin were detected by paper chromatography. Judging from their content in the cuticles, their contribution to pupal colouration seems to be negligible.

(8) These findings are discussed in connexion with the possible action of the hormone which is secreted from prothoracic ganglion and is the immediate determinant of the pupal types.

Acknowledgements—The author wishes to express his gratitude to Prof. Y. Akita of Tokyo Metropolitan University and to Prof. T. Fujii of Tokyo University for their valuable criticism and encouragement. He is also indebted to Prof. N. H. HOROWITZ of California Institute of Technology for reading the manuscript. Thanks are also due to Messrs. K. HAYASHI and T. HIDAKA for their helpful suggestions and discussion, and to Prof. T. HAMA of Keio University for permission to use a spectrophotometer.

REFERENCES

AKINO M. and ISAKA S. (1957) Effect of pteridine and purine derivatives on the metal-catalysed oxidation of 3 : 4 di-hydroxyphenylalanine. *J. Coll. Arts Sci., Chiba Univ.* **2**, 225–233.

BARBER M. E. (1874) *in* WALLACE A. R. (1895) *Natural Selection and Tropical Nature*. Macmillan, London.

CRAMMER J. L. (1948) Paper chromatography of flavin nucleotides. *Nature, Lond.* **161**, 349–350.

GOODWIN T. W. and SRISUKH S. (1948) The biochemistry of locusts—I. The carotenoids of the integument of two locust species (*Locusta migratoria migratorioides* R. and F. and *Schistocerca gregaria* F.) *Biochem. J.* **45**, 263–268.

GOODWIN T. W. and SRISUKH S. (1950) Biochemistry of locusts—V. The green pigment of the haemolymph and integument of solitary locusts (*Locusta migratoria migratorioides* R. and F. and *Schistocerca gregaria* F.). *Biochem. J.* **48**, 199–203.

GOODWIN T. W. (1952) The biochemistry of locust pigmentation. *Biol. Rev.* **27**, 439–460.

GOODWIN T. W. (1952) *The comparative biochemistry of carotenoids*. Chapman and Hall, London.

HIDAKA T. (1956) Recherche sur le déterminisme hormonal de la coloration pupale chez lépidoptères—I. Les effets de la ligature, de l'ablation des ganglions et de l'incision des nerfs chez prépupes et larves âgées de quelque Papilionides. *Annot. zool. jap.* **29**, 69–74.

HIDAKA T. (1957) On the possibility of determination of pupal colour types by some particular 'scents' in the swallowtail, *Papilio xuthus* L. *Kagaku* **27**, 93–94. In Japanese.

ISAKA S. (1952) Inhibitory effect of xanthopterin upon the formation of melanin *in vitro*. *Nature, Lond.* **169**, 74–75.

ISHIZAKI H. and KATO M. (1956) Environmental factors affecting the formation of orange pupa in *Papilio xuthus*. *Mem. Coll. Sci. Kyoto* (B) **23**, 11–19.

JOLY L. (1954) Resultats d'implantations systematiques de *corpora allata* à de jeunes larves de *Locusta migratoria* L. *C. R. Soc. Biol., Paris* **148**, 579–583.

JUNGE H. (1941) Über grüne Insektenfarbstoffe. *Hoppe-Seyl. Z.* **268**, 179–186.

KARRER P. and JUCKER E. (1950) *Carotenoids* (Translated by E. A. Braude). Elsevier, Amsterdam.

LEMBERG R. and LEGGE J. W. (1949) *Hematin compounds and bile pigments*. Interscience, New York.

NICKERSON B. (1954) A possible endocrine mechanism controlling locust pigmentation. *Nature, Lond.* **174**, 357–358.

OHNISHI E. and HIDAKA T. (1955) Effect of environmental factors on the determination of pupal types in some swallowtails, *Papilio xuthus* L. and *P. protenor demetrius* Cr. *Zool. Mag., Tokyo* **65**, 185–187.

OHNISHI E. and HIDAKA T. (1957) Effet des facteurs du milieu ambiant sur la détermination des types de chrysalides de certains papillons diurnes nommés 'queue d'hirondelle'. *Bull. Soc. Hist. nat. Toulouse* **92**, 177–185.

OSHIMA C., SEKI T., and ISHIZAKI H. (1956) Studies on the mechanism of pattern formation in the elythra of lady beetles. *Genetics* **41**, 4–20.

POLONOVSKI M., GONNARD P., and BARIL A. (1951) Action de derives pteriniques et della riboflavine sur la melanogenese *in vitro*. *Enzymologia* **14**, 311–323.

SEVASTOPULO D. G. (1947) The colour relationship between certain pupae and their surroundings. *Proc. R. ent. Soc. Lond.* (A) **23**, 93–95.

UVAROV B. P. (1921) *in* GOODWIN T. W. (1952) The biochemistry of locust pigmentation. *Biol. Rev.* **27**, 439–460.

WIELAND H. and TARTTER A. (1940) Über die Flügelpigmente der Schmetterlinge VIII. Pterobilin, der blaue Farbstoff der Pieridenflügel. *Liebigs Ann.* **545**, 197–208.

WIGGLESWORTH V. B. (1953) *The Principles of Insect Physiology*. Methuen, London.

WOOD T. W. (1867) *in* BRECHER L. (1917) Die Puppenfärbungen des Kohlweisslings, *Pieris brassicae* L. *Arch. EntwMech. Org.* **43**, 88–221.

VOL. 3 1959

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 146 *to* 158. *Pergamon Press Ltd., London. Printed in Great Britain*

THE EFFECTS OF POTASSIUM AND SODIUM IONS ON THE RESTING AND ACTION POTENTIALS OF THE COCKROACH GIANT AXON

TERUO YAMASAKI and TOSHIO NARAHASHI

Laboratory of Applied Entomology, Faculty of Agriculture, University of Tokyo, Tokyo, Japan

(*Received* 10 *November* 1958)

Abstract—The effects of varying the potassium and sodium concentrations in the bathing fluid on the resting and action potentials of the cockroach giant axon have been examined by means of intracellular microelectrodes. Plotting the resting potentials against the logarithms of the external potassium concentrations resulted in a straight line in the concentrations higher than about 20 mM. The decrease in the sodium concentration had no appreciable effect on the resting potential, but effectively depressed the action potential. Plotting the reversed potentials against the logarithms of the external sodium concentrations resulted in a straight line, which diverged only slightly from the theoretically expected straight line having a slope of 59 mV for a tenfold change in sodium. The rate of rise of the action potential was decreased very effectively by the sodium-deficient solution. It can safely be said that the overall result constitutes strong evidence for the essential validity of the sodium theory in the cockroach giant axon.

INTRODUCTION

It has been demonstrated by many investigators that resting potentials of nerve and muscle fibres are dependent upon the external concentration of potassium ions, higher external concentrations causing a depolarization of the membrane (e.g. Hagiwara and Watanabe, 1954; Hodgkin, 1951; Hoyle, 1953, 1954). On the other hand, sodium ions have been known to be indispensable for maintaining excitability, the decrease in the external concentration of sodium ions below a critical level causing a reversible block of conduction (e.g. Hodgkin, 1951; Hoyle, 1953). A number of detailed analyses of membrane behaviour under various ionic environments have led to the conclusion that resting potential is produced by a concentration gradient of potassium ions inside and outside the membrane, whereas an action potential is generated by an entry of sodium ions into the fibre which is followed by an exit of potassium ions from the fibre (Hodgkin, 1951; Hodgkin and Huxley, 1952a, b, c, d; Hodgkin *et al.*, 1949, 1952). This theory of membrane potentials, the sodium theory, is based chiefly on the experiments with squid giant axons, so that it remains to be shown whether the sodium theory is also applicable to the insect axon.

The importance of insect neurophysiology has much increased in recent years, for most of the synthetic insecticides developed recently are nerve poisons and knowledge of the mode of action of such insecticides is urgently required. In the

light of such views it seems worth while to study the electrical properties of insect nerve. The magnitudes of the resting and action potentials of cockroach giant axons have been determined by BOISTEL and CORABOEUF (1954), CORABOEUF and BOISTEL (1955), and YAMASAKI and NARAHASHI (1957). The effects of varying sodium and potassium ion concentrations in the bathing fluid upon resting and action potentials were studied.

METHODS

Male adults of the American cockroach, *Periplaneta americana* L., reared in the laboratory at a constant temperature of about 30°C, were used throughout the experiments. The giant axons in the abdominal nerve cord, which had been described by ROEDER (1948), were used in the present study.

Microelectrodes

The microelectrodes employed were the same type as those used by LING and GERARD (1949) and NASTUK and HODGKIN (1950). They were drawn in a machine from hard glass tubing and filled with 3 M KCl. The microelectrodes having a resistance of 5–15 MΩ were selected for the experiments. The external tip diameters of such microelectrodes were not measured exactly; preliminary observations revealed that they were of the order of 0·5 to 1 μ.

VOL. 3 1959

Recording apparatus

Compensation circuits for improving high frequency responses have so far been devised by several workers (HAAPANEN and OTTOSON, 1954; MACNICHOL and WAGNER, 1954; SOLMS *et al.*, 1953). Since the rate of rise of the action potential in the cockroach giant axon is as high as 550 V/sec (YAMASAKI and NARAHASHI, 1957), it is highly desirable to employ such a compensation device in order to record the true shape and magnitude of the action potential. A slightly modified circuit of the HAAPANEN and OTTOSON type (1954) was used in the present experiments in place of a simple cathode follower circuit which had been used in our previous work (YAMASAKI and NARAHASHI, 1957). This compensation circuit made it possible for the time constant of the rising phase of square pulse applied to fall as short as 16–20 μsec when the microelectrodes having a resistance of 5–15 MΩ were employed. With this device graphical compensations made in our previous work were unnecessary. The grid current in the initial cathode follower stage of this circuit was of the order of 10^{-12}A.

A microelectrode was connected to the input of this compensation circuit by means of a fine Ag-AgCl wire of about 100μ diameter and 10 cm length. An Ag-AgCl rod of about 2 mm diameter and 3 cm length was immersed in the bathing fluid as the indifferent electrode. The compensation circuit was connected to a d.c. amplifier, the output of which was then led to an oscilloscope. Calibration voltage was applied to the recording system through a low resistance which was inserted in the ground lead.

Solutions

The same Ringer's solution as that described in our previous paper was used as a bathing fluid in the initial stage of the present study (YAMASAKI and NARAHASHI, 1957). However, it was found in the course of the present experiments that the axons swell slightly after being kept in the Ringer's solution for a period of several hours. Such swelling is to be expected when the bathing solution is more hypotonic than the haemolymph serum. It has actually been shown that the haemolymph serum of the cockroach is isotonic to 224 mM NaCl (LUDWIG *et al.*, 1957). The components of Ringer's solution were therefore modified in the later stage of the present study, the concentration of sodium ions being

increased. The compositions of the modified Ringer and that previously used (in brackets) are as follows: 214 (159·6) mM Na^+, 3·1 (3·1) mM K^+, 1·8 (1·8) mM Ca^{++}, 216·9 (160·1) mM Cl^-, 0·2 (0·2) mM $H_2PO^-_4$ and 1·8 (1·8) mM HPO^{--}_4. These Ringer's solutions have a pH of 7·2. With the modified solution the axons could be kept unchanged over a long period of time.

The kind of Ringer's solution employed will be described in each section of the results.

Potassium-rich solutions were made by mixing Ringer's solution with the isotonic potassium chloride solution. Potassium-deficient solutions were prepared separately.

Sodium-rich solutions were made by adding solid sodium chloride to Ringer's solution. Sodium-deficient solutions were made by mixing Ringer's solution with the isotonic choline chloride solution or the isotonic glucose solution.

Experimental procedure

An isolated nerve cord was desheathed with fine needles at a small region of the connectives between the fourth and the fifth abdominal ganglia. The nerve preparation was then immersed and fixed in the bathing fluid in a small Perspex chamber. The anterior region of the nerve cord from the metathoracic ganglion to the third abdominal ganglion was located out of the bathing fluid and electrical stimulation was applied to this region. The microelectrode was inserted slowly into the desheathed region of the nerve cord at an angle of approximately 45° to the longitudinal axis of the nerve cord.

Two methods were used for exchanging solution. In order to keep the microelectrode inserted in an axon, the bathing fluid had to be substituted for a test solution by slow and gentle continuous perfusion. A test solution was poured slowly into the nerve chamber, and an outlet attached on the side wall of the chamber allowed the level of bathing fluid to be kept constant during perfusion. The perfusion was continued until the bathing fluid was completely replaced by the test solution, the time required for the complete replacement being checked using the solution containing dye in the other set of the experiments. An alternative method depended on a simple exchange of the bathing fluid with a test solution using a pipette, so that the microelectrode which had once been withdrawn from the axon had to be impaled in other axons after replacing the bathing fluid. In any case the measurements of membrane potentials were made from five to ten minutes after substituting the bathing fluid for the test solution.

All the experiments were conducted at room temperatures ranging from 23°C to 29°C. The temperature during any single series of experiments was never changed by over 1°C.

RESULTS

Normal resting and action potentials

The magnitudes of the resting potential, action potential, reversed potential, and after-positivity, and the rates of rise and fall of the action potential are shown in Table 1. Only the values obtained from the axons immersed in the modified Ringer's solution are listed in that Table. A typical picture of the action potential is given in Fig. 1. A total of 47 successful impalements of the axons immersed in the unmodified Ringer's solution resulted in the following average values of the membrane potentials (ranges of the values): resting potential 70·1 mV (64·5–79·0 mV), action potential 86·9 mV (80·1–103·7 mV), reversed potential 16·8 mV (10·7–26·4 mV), after-positivity 3·6 mV (0–7·2 mV), maximum rate of rise of action potential 926 V/sec (650–1455 V/sec), and maximum rate of fall 397 V/sec (264–534 V/sec).

No significant difference was found for the resting potential, the after-positivity, and the rate of fall of the action potential between the two kinds of Ringer's

VOL.
3
1959

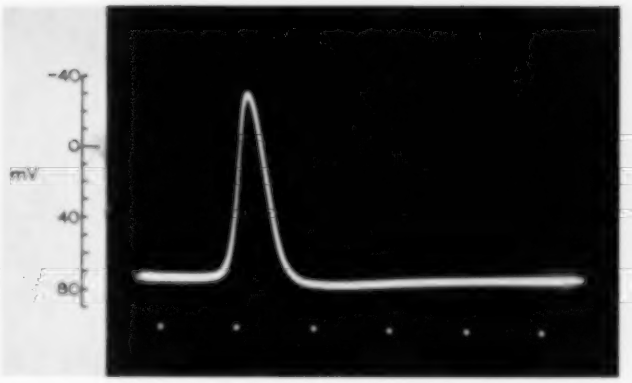


FIG. 1. An action potential of the cockroach giant axon recorded by means of an intracellular microelectrode. Voltage calibration, 10 mV. Time marker, 0·5 msec 26°C.

solution, whereas the magnitude and the rate of rise of the action potential were somewhat higher when the modified Ringer's solution was used. These results are to be expected and will be discussed later.

TABLE 1—THE MEMBRANE POTENTIALS OF THE GIANT AXON OF THE COCKROACH

No.	Temperature (°C)	Resting potential (mV)	Action potential (mV)	Reversed potential (mV)	After-positivity (mV)	Maximum rate of rise of action potential (V/sec)	Maximum rate of fall of action potential (V/sec)
1	26	71·5	99·1	27·6	4·6	1162	410
2	26	66·2	90·9	24·7	4·7	905	305
3	26	69·2	90·9	21·7	3·7	1055	338
4	26	73·1	100·4	27·3	3·0	1309	511
5	26	69·2	93·2	24·0	5·7	1228	547
6	26	72·0	100·8	28·8	3·4	1466	486
7	26	73·0	97·6	24·6	0	1089	377
8	26	68·2	90·2	22·0	3·4	948	314
9	28	71·5	97·4	25·9	1·5	1349	504
10	28	65·8	89·9	24·1	4·1	1176	470
11	28	70·0	92·4	22·4	3·6	1552	426
12	28	78·3	101·2	22·9	0	1475	499
13	28	75·1	98·7	23·6	1·9	1630	595
14	26	63·3	91·1	27·8	5·2	1055	320
15	26	70·2	90·5	20·3	4·0	940	294
16	24·5	67·8	89·2	21·4	1·4	804	230
17	24·5	68·1	89·6	21·5	4·3	905	331
18	24·5	69·1	91·7	22·6	5·5	807	267
19	24·5	66·0	95·4	29·4	8·0	1143	320
20	24·5	69·5	97·5	28·0	4·8	966	298
21	24·5	66·0	89·0	23·0	4·5	879	301
22	24·5	72·6	97·6	25·0	1·8	998	378
23	24·5	74·0	99·2	25·2	1·8	874	351
24	24·5	78·0	94·3	19·9	1·8	987	301
Mean	25·9	70·3	94·5	24·2	3·4	1113	382

Effect of varying the external concentration of potassium ions on resting potential

All the experiments were carried out using the unmodified Ringer's solution as a bathing fluid except for 220 mM potassium ions. Plotting the average values of the resting potentials against the logarithms of the external potassium concentrations resulted in a curve illustrated by Fig. 2. In the concentrations higher than about 20 mM the observed values fell close to a straight line. In the concentrations less than 3·1 mM no appreciable difference was found among the observed values.

Although no detailed observations were made on the change in action potential under various concentrations of potassium ions, it was usually observed in the course of the above-mentioned experiments that residual small action potentials appear on stimulation even in the severely depolarized axons. The nature and mode of production of such residual action potentials will be discussed later.

VOL. 3 1959

Effect of varying the external concentrations of sodium ions on resting and action potentials

In order to compare the membrane behaviour of the cockroach giant axons under various external concentrations of sodium ions with those of giant axons of the squid, *Loligo*, the present experiments were carried out along the same line as the experiments with squid axons (HODGKIN and KATZ, 1949a).

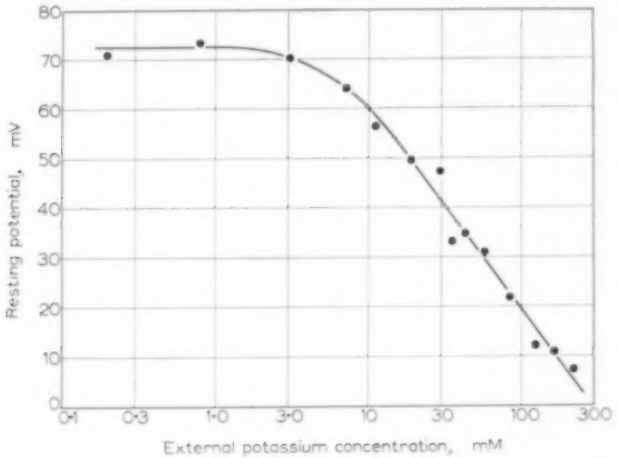


FIG. 2. Effect of varying the external concentration of potassium (abscissa) upon resting potential (ordinate).

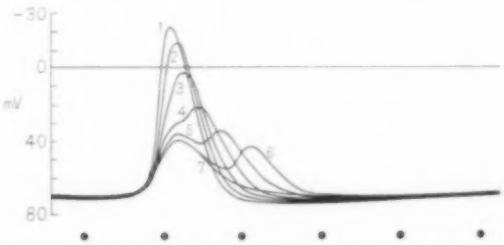


FIG. 3. Change in action potential after perfusing the axon with the isotonic glucose solution. Record 1 indicates a normal action potential recorded immediately before perfusion. 2–7 indicate 4 min, 4 min 30 sec, 5 min, 5 min 30 sec, 5 min 45 sec, and 6 min after the start of perfusion respectively. The unmodified type of Ringer's solution (159·6 mM sodium) was used. Voltage calibration, 10 mV. Time marker, 0·5 msec 24°C.

(a) *Effect of perfusing the sodium-free solution.* The effect of sodium-free solution was examined by allowing the axon to be perfused by an isotonic glucose solution. The unmodified Ringer's solution was used in this case. The changes in action potential are shown in Fig. 3. Here the action potentials were traced from the same base-line, because it was found later that the resting potential undergoes little change upon varying the external sodium concentration. Record 1 shows a normal

action potential of the axon immersed in Ringer's solution. After taking this record the perfusion of the isotonic glucose solution was started. The action potential progressively decreased in magnitude and became double-peaked in appearance. The second peak delayed progressively and finally disappeared. The first peak further decreased in magnitude only very slowly and also disappeared completely when the axon was allowed to be in contact with the sodium-free solution for a long period of time. When the normal Ringer's solution was restored immediately after the disappearance of the second peak, the action potential recovered to a value which was close to that initially observed.

It seems convenient to consider here the mechanism for the production of a double-peaked action potential. The fact that the residual deflexion or the first peak has a lower amplitude and longer duration than the normal action potential strongly suggests the possibility that this residual deflexion is due to electrotonic spread from the part of the axon other than the point of impalement. Since the nerve preparation was only partly desheathed to make the impalement of the microelectrode easier, it is reasonable to suppose the origin of such a residual deflexion to be near the boundary between the intact and the desheathed part of the nerve if the sheath behaved as a strong barrier against the penetration of sodium and potassium ions.

VOL. 3 1959

For these reasons some observations were made on whether the nerve sheath was a barrier or not. The undesheathed nerve preparation was immersed in the isotonic glucose solution, and its excitability was examined from time to time by lifting the nerve up from the solution and observing the action potentials on stimulation by external electrodes. Immediately after applying the isotonic glucose, the magnitude of the action potential increased somewhat, which was undoubtedly due to the decrease in short-circuit resistance between the two recording electrodes. The action potential was kept unchanged for a long period of time after the application of the isotonic glucose, but a block was finally caused. The time necessary for the complete block was very different in each preparation, being of the order of one to several hours. Thus it was clearly demonstrated that the nerve sheath behaves as a strong barrier against the penetration of sodium ions, which is in good agreement with the experiments of TWAROG and ROEDER (1957) on the cockroach nerve.

In view of such evidence it seems reasonable to consider that the residual deflexion is almost certainly due to electrotonic spread from the part of the axon where the sheath is left intact. The residual deflexions observed with the potassium-rich solution would be explained similarly.

There is some possibility of saltatory conduction beyond the desheathed and blocked region of the axon. If this did occur one could record electrotonically two action potentials generated before and after saltatory conduction.

For these reasons the action potentials showing two distinct peaks are scarcely worth considering because they do not generate at the point of impalement. Therefore, the measurements were made on the action potentials which did not show two distinct peaks. Careful observations of series of records such as Fig. 3 revealed

that although the rate of rise of the action potential decreased rapidly when the magnitude of the action potential decreased, the rate of fall of the action potential decreased much less. These events are illustrated by Fig. 4. A comparable decrease in the maximum rate of fall with that in the magnitude implies the possibility that the former is an inevitable consequence of the latter. On the other hand, an additional mechanism must be involved in the decrease in the maximum rate of rise.

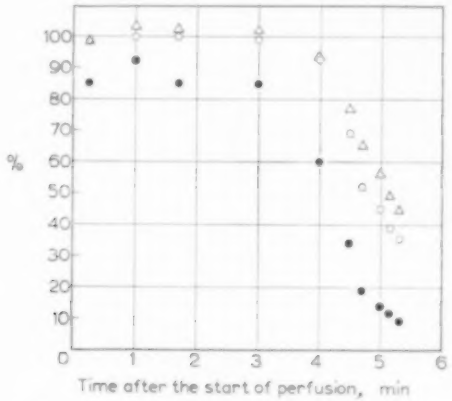


FIG. 4. Decrease in magnitude (triangles) of action potential, and in rates of rise (solid circles) and fall (hollow circles) of action potential after perfusing the axon with the isotonic glucose solution. The normal values obtained immediately before perfusion were taken as 100%. These measurements were made on the same experiment shown in Fig. 3.

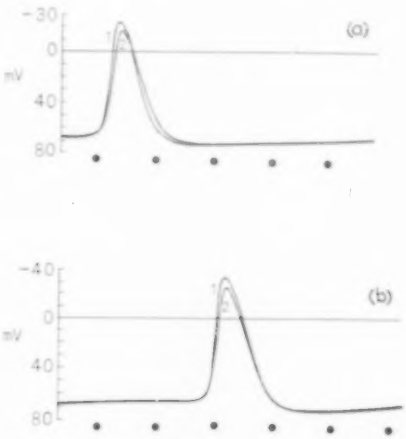


FIG. 5. Changes in action potential after perfusing the axon with sodium-deficient (107 mM) (a) and the sodium-rich (315 mM) (b) Ringer's solution. Records 1 and 2 indicate before and after perfusion respectively. The modified Ringer's solution (214 mM sodium) was used as a normal bathing solution. (a) and (b) were obtained from different axons. Voltage calibration, 10 mV. Time marker, 0·5 msec 24·5°C.

Another feature to be noted is that the after-positivity is only slightly affected by the sodium-free solution.

(b) *Effect of varying the sodium concentration.* In this series of experiments choline chloride was exclusively used for maintaining the isotonicity of the sodium-deficient Ringer's solution. Choline chloride was shown to be inert in the case of squid giant axon (HODGKIN and KATZ, 1949a). The modified Ringer's solution was mainly employed as a bathing fluid.

Examples of the changes in action potential following treatment of the axon with the low-sodium and high-sodium Ringer's solutions are illustrated by Fig. 5. It can be seen that the magnitude and the rate of rise of the action potential are

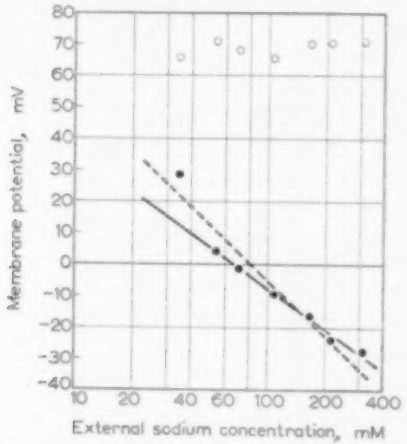


FIG. 6. Effect of varying the external concentrations of sodium (abscissa) upon resting potential (hollow circles) and reversed potential (solid circles) (ordinate). The broken line is drawn according to equation (2), which has a slope of 59 mV for a tenfold change in sodium concentration. The solid line is drawn through the observed values of the reversed potential excluding the lowest value. The modified Ringer's solution was used as a normal bathing solution.

dependent upon the external sodium concentration, the low-sodium solution causing the decrease and the high-sodium solution causing the increase. Such behaviour of the active membrane becomes clearer when the average membrane potentials are plotted against the logarithms of the external sodium concentrations (Fig. 6). The average values of the resting potential are also included in this figure.

According to the sodium theory (HODGKIN, 1951; HODGKIN and KATZ, 1949a), the potential difference across the active membrane should be given by the equilibrium potential for sodium, E_{Na}:

$$E_{Na} = \frac{RT}{F} \log_e \frac{[Na]_i}{[Na]_0}, \qquad (1)$$

where R is the gas constant, T is the absolute temperature, F is the Faraday, and $[Na]_i$ and $[Na]_0$ are sodium concentrations, or, more strictly, sodium activities,

in the axoplasm and external solution respectively. Hence the change in reversed potential, ΔE, which results from an alteration of external sodium should be given by equation:

$$\Delta E = E_t - E_R = \frac{RT}{F} \log_e \frac{[Na]_R}{[Na]_t}, \quad (2)$$

where E_t and E_R are the membrane potentials measured in test and Ringer's solutions respectively, and $[Na]_t$ and $[Na]_R$ are the sodium concentrations in test and Ringer's solutions respectively.

A slope of 59 mV for a tenfold change in sodium concentration was therefore drawn by a broken line, in Fig. 6. Since the average temperature of the present experiments was approximately 26°C, the value of 59 mV was adopted instead of 58 mV. Although all the data excluding the lowest sodium concentration fell close to the solid line rather than to the theoretically expected broken line, the divergences of the observed values from the theoretical line were not so marked. The active membrane potential for 35 mM sodium diverged considerably from the solid line. This result seems to be due partly to the fact that the action potentials were liable to decrease in magnitude progressively when the sodium concentration was as low as about 35 mM.

The magnitude of the resting potential underwent little change even when the external sodium concentration was varied over a wide range.

DISCUSSION

The membrane potentials measured with the unmodified Ringer's solution at 19°C and with their distortion compensated graphically are as follows: resting potential 64·5 mV, action potential 92·2 mV, reversed potential 27·7 mV, after-positivity 2·8 mV, maximum rate of rise of action potential 548 V/sec, and maximum rate of fall 278 V/sec (YAMASAKI and NARAHASHI, 1957). Hence the present experiments give slightly higher average values for the resting potential, the after-positivity, and the maximum rates of rise and fall of the action potential, and give a slightly lower average value for the action potential. The higher rates of rise and fall seem to be reasonable, for the temperatures in the present experiments are considerably higher than those in the previous ones; a similar event was observed in squid giant axon (HODGKIN and KATZ, 1949b). It seems safe to assume that the present results obtained by electrical compensations for improving high frequency responses, especially with regard to the action potential, are more reliable than the previous ones obtained by graphical compensations.

The measurements of the membrane potentials of the cockroach giant axons were also made by BOISTEL and CORABOEUF (1954) and CORABOEUF and BOISTEL (1955). They obtained the average resting potential of 78 mV (60–89 mV) and the average action potential of 85 mV (65–92 mV) using the Ringer's solution which contained almost the same ionic compositions as that of our unmodified type. The average value of the action potentials they obtained is in good agreement with that of the present experiments conducted with the unmodified Ringer's solution, while the range of their values is somewhat wider and shifts to the lower

side. The average value of the resting potentials they obtained is somewhat higher and the range of their values is somewhat wider than those of the present experiments.

Under such considerations as these, it would be worthwhile to compare the more reliable present values with the theoretical ones, though such a comparison has been attempted in our previous paper using the less reliable observed values (YAMASAKI and NARAHASHI, 1957).

The resting potential is related to the relative concentration of potassium ions thus:

$$E_k = \frac{RT}{F} \log_e \frac{[K]_i}{[K]_0}, \tag{3}$$

where E_k is the equilibrium potential for potassium, and $[K]_i$ and $[K]_0$ are potassium concentrations inside and outside the nerve respectively (HODGKIN, 1951). The content of potassium in the sheathed nerve cord was determined as 140 mM/l. of tissue water (TOBIAS, 1948). Using this figure the calculations from the equation (3) result in the equilibrium potential for potassium of 97·7 mV under the present experimental conditions. This value, however, must be underestimated; the potassium concentration of the haemolymph serum in the cockroach is found to be much less than that of the nerve cord, i.e., 17·3 mM/l. of tissue water (TOBIAS, 1948), which results in the actual concentration of potassium in the roach nerve fibre being higher than 140 mM. Hence the discrepancy between the observed and the calculated resting potentials amounts to over 27·4 mV.

The high equilibrium potential for potassium ions is likely to be partly responsible for the production of the after-positivity which follows the spike action potential (FRANKENHAEUSER and HODGKIN, 1956; HODGKIN, 1951; HODGKIN and KEYNES, 1955). It seems reasonable to suppose that the resting membrane of the cockroach giant axon is not exclusively permeable to potassium ions but slightly permeable to other ions such as sodium, so that the observed resting potentials become lower than the calculated potential. It follows that the increase in membrane permeability to potassium ions during the falling phase of the action potential causes the membrane to approach the equilibrium potential for potassium ions. This view is consistent with the finding that the sodium-deficient Ringer's solutions are without effect on the magnitude of the positive phase even though they affect the reversed potential appreciably. However, the after-positivity does not reach the potential difference expected from the theoretical equilibrium potential for potassium ions. Therefore it seems probable that an unselected barrier between the excitable membrane and the external solution prohibits a rapid movement of potassium ions to some extent as has been suggested by FRANKENHAEUSER and HODGKIN (1956).

The behaviour of the resting membrane under various external concentrations of potassium ions is essentially similar to that observed with both nerves and muscles of other animals (e.g. BURGEN and TERROUX, 1953; CURTIS and COLE, 1942; FATT and KATZ, 1953; HAGIWARA and WATANABE, 1954; HARRIS and MARTINS-FERREIRA, 1955; HODGKIN, 1951; HOYLE, 1952, 1953, 1954; WEIDMANN, 1955).

VOL. 3 1959

The curve for resting potential/potassium is nearly a straight line in the higher range of potassium concentrations. This straight line has a slope of 42 mV for a tenfold change in potassium concentration, and diverges slightly from the expected slope of 59 mV based on the equation (3). However, such divergence seems to be reasonable, for the resting membrane is considered to be, not exclusively, but mainly permeable to potassium ions as mentioned before. All that can be said is that the resting membrane of the cockroach giant axon behaves quite like those of other nerves and muscles when the external potassium concentration is altered.

The concentration of sodium in the sheathed roach nerve cord is estimated to be 83·9 mM/l. of tissue water (TOBIAS, 1948). The calculations of the magnitude of the reversed potential from the equation (1) result in the value of 24·0 mV when the external sodium concentration of 214 mM (the modified Ringer's solution) is used for calculation. This theoretical value is in good agreement with the observed value of 24·2 mV. However, this theoretical value must be slightly underestimated, because the sodium concentration of the cockroach haemolymph serum is slightly higher than that of the nerve cord, i.e., 107 mM (TOBIAS, 1948). The slightly higher value of the calculated reversed potential is likely to be reasonable, because this figure gives only the maximum value to be reached and also because the slight permeability of the ions other than sodium may take part in the peak of the action potential to some extent.

The behaviour of the active membrane of the cockroach giant axon under a variety of external sodium concentrations is quite similar to that observed with squid giant axon with regard to the magnitude of the reversed potential and after-positivity and in the rates of rise and fall of the action potential (HODGKIN and KATZ, 1949a). The present results are also in good agreement with those reported with other nerves and muscles (DRAPER and WEIDMANN, 1951; HAGIWARA and WATANABE, 1955; HUXLEY and STÄMPFLI, 1951; NASTUK and HODGKIN, 1950; WEIDMANN, 1955). The straight line for reversed potential/sodium to which almost all the observed values fell diverges slightly from the expected slope of 59 mV based on the equation (2), but this divergence is not so serious. A more marked decrease in the rate of rise of the action potential than those in the rate of fall and in the magnitude when the axon comes into contact with the sodium-deficient Ringer's solution strongly suggests the possibility that the entry of sodium ions plays an important rôle in the rising phase of the action potential, because the rate of rise is known to be correlated with the rate of entry of sodium ions into the squid axon (HODGKIN and KATZ, 1949a). In the light of such views, it can safely be said that the overall result constitutes strong evidence for the essential validity of the sodium theory in the cockroach giant axon.

SUMMARY

The effects of varying the potassium and sodium concentrations in the bathing fluid on the resting and action potentials of the cockroach giant axon have been examined by means of intracellular microelectrodes.

The modified Ringer's solution for the cockroach axon was described. This contains a greater amount of sodium ions than the ordinary roach saline.

The mean values of the membrane potentials from the axons immersed in the modified Ringer's solution were as follows: resting potential 70·3 mV, action potential 94·5 mV, after-positivity 3·4 mV, maximum rate of rise of action potential 1113 V/sec, and maximum rate of fall 382 V/sec.

The increase in potassium concentration caused a depolarization. Plotting the resting potentials against the logarithms of the external potassium concentrations resulted in a straight line in the concentrations higher than about 20 mM. In the concentrations less than 3·1 mM no appreciable change in resting potential was observed.

The decrease in sodium concentration had no appreciable effect on the resting potential, but effectively depressed the action potential and finally brought about a block. Plotting the reversed potentials against the logarithms of the external sodium concentrations resulted in a straight line. This straight line diverged only slightly from the theoretically expected straight line having a slope of 59 mV for a tenfold change in sodium. The rate of rise of the action potential decreased markedly more than both the rate of fall and the magnitude of the action potential when the sodium concentration was reduced.

These results were discussed in the light of the sodium theory. It can safely be said that the overall result constitutes strong evidence for the essential validity of the sodium theory in the cockroach giant axon.

VOL. 3 1959

REFERENCES

Boistel J. and Coraboeuf E. (1954) Potentiel de membrane et potentiels d'action de nerf d'insecte recueillis à l'aide de microélectrodes intracellulaires. *C. R. Acad. Sci., Paris* **238**, 2116–2118.

Burgen A. S. V. and Terroux K. G. (1953) The membrane resting and action potentials of the cat auricle. *J. Physiol.* **119**, 139–152.

Coraboeuf E. and Boistel J. (1955) Quelques aspects de la microphysiologie nerveuse chez les insectes. *Colloq. Internat. Cent. Nat. Rech. Sci. No.* 67, *Microphysiologie Comparée des Éléments Excitables*, 57–72.

Curtis H. J. and Cole K. S. (1942) Membrane resting and action potentials from the squid giant axon. *J. cell. comp. Physiol.* **19**, 135–144.

Draper M. H. and Weidmann S. (1951) Cardiac resting and action potentials recorded with an intracellular electrode. *J. Physiol.* **115**, 74–94.

Fatt P. and Katz B. (1953) The electrical properties of crustacean muscle fibres. *J. Physiol.* **120**, 171–204.

Frankenhaeuser B. and Hodgkin A. L. (1956) The after-effects of impulses in the giant nerve fibres of *Loligo*. *J. Physiol.* **131**, 341–376.

Haapanen L. and Ottoson D. (1954) A frequency compensated input unit for recording with microelectrodes. *Acta physiol. scand.* **32**, 271–280.

Hagiwara S. and Watanabe A. (1954) Action potential of insect muscle examined with intra-cellular electrode. *Jap. J. Physiol.* **4**, 65–78.

Hagiwara S. and Watanabe A. (1955) The effect of tetraethylammonium chloride on the muscle membrane examined with an intracellular microelectrode. *J. Physiol.* **129**, 513–527.

Harris E. J. and Martins-Ferreira H. (1955) Membrane potentials in the muscles of the South American frog, *Leptodactylus ocellatus*. *J. exp. Biol.* **32**, 539–546.

HODGKIN A. L. (1951) The ionic basis of electrical activity in nerve and muscle. *Biol. Rev.* **26**, 339–409.

HODGKIN A. L. and HUXLEY A. F. (1952a) Current carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. *J. Physiol.* **116**, 449–472.

HODGKIN A. L. and HUXLEY A. F. (1952b) The components of membrane conductance in the giant axon of *Loligo*. *J. Physiol.* **116**, 473–496.

HODGKIN A. L. and HUXLEY A. F. (1952c) The dual effect of membrane potential on sodium conductance in the giant axon of *Loligo*. *J. Physiol.* **116**, 497–506.

HODGKIN A. L. and HUXLEY A. F. (1952d) A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.* **117**, 500–544.

HODGKIN A. L. and KATZ B. (1949a) The effect of sodium ions on the electrical activity of the giant axon of the squid. *J. Physiol.* **108**, 37–77.

HODGKIN A. L. and KATZ B. (1949b) The effect of temperature on the electrical activity of the giant axon of the squid. *J. Physiol.* **109**, 240–249.

HODGKIN A. L. and KEYNES R. D. (1955) The potassium permeability of a giant nerve fibre. *J. Physiol.* **128**, 61–88.

HODGKIN A. L., HUXLEY A. F., and KATZ B. (1949) Ionic currents underlying activity in the giant axon of the squid. *Arch. Sci. Physiol.* **3**, 129–150.

HODGKIN A. L., HUXLEY A. F., and KATZ B. (1952) Measurement of current-voltage relations in the membrane of the giant axon of *Loligo*. *J. Physiol.* **116**, 424–448.

HOYLE G. (1952) High blood potassium in insects in relation to nerve conduction. *Nature, Lond.* **169**, 281–283.

HOYLE G. (1953) Potassium ions and insect nerve muscle. *J. exp. Biol.* **30**, 121–135.

HOYLE G. (1954) Changes in the blood potassium concentration of the African migratory locust (*Locusta migratoria migratorioides* R. and F.) during food-deprivation, and the effect on neuromuscular activity. *J. exp. Biol.* **31**, 260–270.

HUXLEY A. F. and STÄMPFLI R. (1951) Effect of potassium and sodium ions on resting and action potentials of single myelinated nerve fibres. *J. Physiol.* **112**, 496–508.

LING G. and GERARD R. W. (1949) The normal membrane potential of frog sartorius fibres. *J. cell. comp. Physiol.* **34**, 383–396.

LUDWIG D., TRACEY S. K. M., and BURNS M. (1957) Ratios of ions required to maintain the heart beat of the American cockroach, *Periplaneta americana* Linnaeus. *Ann. ent. Soc. Amer.* **50**, 244–246.

MACNICHOL E. F. and WAGNER H. G. (1954) A high impedance input circuit suitable for electrophysiological recording from micropipette electrodes. *Nav. med. res. Inst.* **12**, 97–118.

NASTUK W. L. and HODGKIN A. L. (1950) The electrical activity of single muscle fibres. *J. cell. comp. Physiol.* **35**, 39–74.

ROEDER K. D. (1948) Organization of the ascending giant fiber system in the cockroach (*Periplaneta americana*). *J. exp. Zool.* **108**, 243–261.

SOLMS S. J., NASTUK W. L., and ALEXANDER J. T. (1953) Development of a high fidelity preamplifier for use in the recording of bioelectric potentials with intracellular electrodes. *Rev. sci. Inst.* **24**, 960–967.

TOBIAS J. M. (1948) Potassium, sodium and water interchange in irritable tissues and haemolymph of an omnivorous insect, *Periplaneta americana*. *J. cell. comp. Physiol.* **31**, 125–142.

TWAROG B. M. and ROEDER K. D. (1957) Pharmacological observations on the desheathed last abdominal ganglion of the cockroach. *Ann. ent. Soc. Amer.* **50**, 231–237.

WEIDMANN S. (1955) The effect of the cardiac membrane potential on the rapid availability of the sodium-carrying system. *J. Physiol.* **127**, 213–224.

YAMASAKI T. and NARAHASHI T. (1957) Studies on the mechanism of action of insecticides (XIV). Intracellular microelectrode recordings of resting and action potentials from the insect axon and the effects of DDT on the action potential. *Botyu-Kagaku* **22**, 305–313.

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 159 *to* 189. *Pergamon Press Ltd., London. Printed in Great Britain*

DIE AUSLÖSUNG DER UMFÄRBUNG DURCH DAS HÄUTUNGSHORMON BEI *CERURA VINULA* L. (LEPIDOPTERA, NOTODONTIDAE)*†

DETLEF BÜCKMANN**

Zoologisches Institut der Johannes Gutenberg-Universität, Mainz

(*Received* 16 *November* 1958)

Abstract—The green larvae of *Cerura vinula* L. turn dark red when they are going to spin their cocoon. A red ommochrome pigment is formed in the epidermal cells and one day later in the fat body. The epidermal processes of the pupal moult begin only five days later. The colour change can be inhibited by a ligature. Only the part of the body anterior to the ligature will redden. Double ligaturing experiments show that the colour change is caused by a thoracic centre, which has to be activated by a cephalic centre, probably the brain. Extracts of the moulting hormone α-ecdyson were injected into green abdomens of ligatured animals. Small doses (66 *Calliphora* units) caused reddening of the epidermis. 330 *Calliphora* units caused reddening of the fat body only, and large doses (3300–6600 units) caused a pupal moult without colour change. It is concluded that both the colour change and the pupal moult are caused by the same hormone, ecdyson, but by different concentrations of it. The normal course of events is probably brought about by a slow increase of hormone concentration. In young larvae premature colour change could be evoked by decapitation. Probably it is normally prevented by the juvenile hormone of the corpora allata.

VOL. 3 1959

ZUSAMMENFASSUNG

Die Raupen von *Cerura vinula* L. färben sich um, wenn sie ihr Futter verlassen, um ihren Kokon zu spinnen. Die hellgrünen Raupen werden innerhalb weniger Stunden dunkelrot gefärbt durch Ommochromfarbstoffe, die zunächst in der Epidermis und dann auch im Fettkörper abgelagert werden. Die Auslösung dieses Farbwechsels wird untersucht. (Alle folgenden Zeitangaben gelten für eine Zuchttemperatur von 25°C.)

(1) Um zur Umfärbung in der Lage zu sein, benötigen die Raupen eine Freßperiode von 4 Tagen nach der letzten Larvenhäutung. Unabhängig davon, ob sie nach dieser Zeit noch weiter fressen bis zum Erreichen des normalen End-Körpergewichtes oder nicht, erfolgt die Umfärbung durchschnittlich 5,6 Tage nach der letzten Larvenhäutung. Die Auslösung der Umfärbung erfolgt also unabhängig vom Zeitpunkt der Beendigung der Nahrungsaufnahme und von der erreichten Körpergrösse.

(2) Rechtzeitige Abschnürung des Kopfes oder Enthirnung können die Umfärbung verhindern. Wird zusätzlich durch eine zweite Ligatur das Abdomen vom Thorax getrennt, so färbt sich in manchen Fällen noch der Thorax um, nicht

* W. v. Buddenbrock zum 75. Geburtstag gewidmet.

† Habilitationsschrift, Mainz, 1957, gekürzte Fassung. Gedruckt mit einer Beihilfe der Deutschen Forschungsgemeinschaft.

** Jetzt: Zoologisches Institut der Universität, Göttingen.

aber das Abdomen. Daraus wird geschlossen, dass die Umfärbung vom Gehirn ausgelöst wird, aber nicht direkt, sondern durch die Aktivierung eines umfärbungsauslösenden Zentrums im Thorax.

(3) Nach einfacher Durchschnürung hinter dem Thorax färbt sich das vordere Körperstück immer um, das hintere aber nur, wenn die Umfärbung innerhalb von 12 Stunden nach der Schnürung erfolgt, und zwar in 50 Prozent dieser Fälle. In manchen Fällen kommt es zu einer Teilumfärbung des hinteren Körperstücks. Daraus wird geschlossen, dass die Umfärbung durch einen stofflichen Faktor ausgelöst wird, dessen Ausbreitung durch eine Ligatur verhindert werden kann und der durchschnittlich etwa 6 Stunden vor der Umfärbung vom umfärbungsauslösenden Zentrum im Thorax abgegeben wird.

(4) Durchschnürten Raupen, bei denen sich nur der Thorax umgefärbt hatte, wurden verschiedene Dosen des Häutungshormons α-Ecdyson in das grüngebliebene isolierte Abdomen injiziert. Geringe Dosen (66 CE) bewirkten Umfärbung nur der Epidermis, mittlere Dosen (330 CE) bewirkten Umfärbung nur des Fettkörpers, und starke Dosen (6600 CE) bewirkten Puppenhäutung ohne Umfärbung. Daraus wird geschlossen, dass die Umfärbung und die Puppenhäutung durch verschiedene Konzentrationen desselben Hormons, Ecdyson, ausgelöst werden. Das umfärbungsauslösende Zentrum in Thorax muss demnach der Bildungsort dieses Hormons sein, also die Prothoraxdrüse. Die Aufeinanderfolge von Umfärbung der Epidermis, Umfärbung des Fettkörpers und Puppenhäutung in der normalen Entwicklung kommt wahrscheinlich durch eine langsam ansteigende Hormonkonzentration zustande.

(5) In Larven des vorletzten Stadiums liess sich eine vorzeitige Umfärbung auslösen durch Dekapitation. Wahrscheinlich wird normalerweise die Umfärbung in den jungen Larven verhindert durch das Juvenilhormon der Corpora allata.

(A) EINLEITUNG UND PROBLEMSTELLUNG

Die Umfärbung der Raupen des grossen Gabelschwanz, *Cerura vinula* L. gehört zu den auffälligsten Farbwechselvorgängen im Tierreich. Schon deshalb verdient sie eine Untersuchung ihrer Ursachen. Besondere Bedeutung aber gewinnt sie durch ihren offensichtlichen Zusammenhang mit der Metamorphose. Die Untersuchung der Umfärbung liess zugleich wichtige Aufschlüsse über Probleme der Physiologie der Metamorphose erhoffen.

Diese Hoffnung gründete sich auf die Ergebnisse der bisherigen Untersuchungen über Verlauf und Auslösung der Umfärbung (BÜCKMANN, 1952, 1953): Die Umfärbung erfolgt im letzten Larvenstadium, und zwar stets gleichzeitig mit dem Verlassen des Futters und dem Kokonbau, obgleich der Zeitabstand zwischen der letzten Larvenhäutung und diesen Verhaltensweisen individuell schwankt. Die fressende Raupe im letzten Stadium ist hellgrün mit einer braunen, weissumränderten, rautenförmigen Zeichnung auf dem Rücken (Abb. 1a). Wenn die Raupe das Futter verlassen hat, um ihren Kokon zu bauen, färbt sie sich innerhalb von 8 Stunden dunkelrot (Abb. 1b). Dieses Rotwerden der Raupe wurde als "Umfärbung" bezeichnet. Unter derselben Bezeichnung erwähnen CASPARI und

PLAGGE (1935) einen ähnlichen Vorgang bei *Sphinx ligustri*. Allerdings verläuft die Umfärbung von *Sphinx ligustri* bedeutend weniger intensiv als diejenige von *Cerura vinula* und erfasst nur den dorsalen Teil des Abdomens. Auch bei anderen Lepidopterenarten sind Farbänderungen vor der Verpuppung zu beobachten (s.z.B. KÜHN und PIEPHO 1936).

Die Umfärbung von *Cerura vinula* beruht auf der Einlagerung eines roten Pigmentes in die Epidermis und in den Fettkörper. Sie ist deshalb als morphologischer Farbwechsel bezeichnet worden (SCHARRER, 1954). Das rote Pigment erwies sich im histochemischen Test als ein Ommatin. Dieses Ergebnis ist inzwischen durch chemische Untersuchungen bestätigt worden (BÜCKMANN, BIEKERT, LINZEN und NEUBERT, unveröffentlicht). Der Umfärbung folgen weitere Veränderungen in Farbe und Form der Raupe. Diese bestehen vor allem in einer Entfärbung der Epidermis und, 5–6 Tage nach der Umfärbung, in der Umwandlung in die Vorpuppe. Erst zu diesem Zeitpunkt setzen in der Epidermis die Häutungsvorgänge ein, die von KÜHN und PIEPHO (1938) als Häutung im weiteren Sinne bezeichnet worden sind. Sie beginnen mit einer Mitosenperiode. Erst etwa 5 Tage später erfolgt die Häutung zur Puppe. Während der ganzen Entwicklung von der Umfärbung bis zur Puppenhäutung ändert sich die Menge und die Zusammensetzung der Ommochromfarbstoffe im Körper. Diese Veränderungen konnten neuerdings quantitativ verfolgt werden (BÜCKMANN, BIEKERT, LINZEN, und NEUBERT, unveröffentlicht).

Die Umfärbung steht also am Beginn einer grossen Reihe von morphologischen und biochemischen Veränderungen, welche die Verpuppung einleiten. Sie sind für die Verpuppung charakteristisch und fehlen bei den Larvenhäutungen. Deshalb ist zu vermuten, dass sie eine physiologische Vorbereitung der Verpuppung und der Metamorphose sind. Sie beginnen schon lange vor den epidermalen Häutungsvorgängen und vor der kritischen Periode, in der die Puppenhäutung hormonal ausgelöst wird. Sie sind also die ersten fassbaren Veränderungen, die das Ende des Larvenlebens und den Beginn der Entwicklung zur Imago ankündigen und vorbereiten.

Unter diesen Veränderungen ist die Umfärbung besonders hervorgehoben. Sie ist nicht nur die zeitlich erste sondern auch die auffälligste. Sie verläuft innerhalb weniger Stunden sehr intensiv, sodass sie sehr leicht und genau zu beobachten und zu registrieren ist. Sie fällt zusammen mit dem Verlassen des Futters und dem Kokonbau, was für eine gemeinsame Auslösung durch dasselbe Aktionssystem spricht. Sie ist ausserdem histologisch und sogar chemisch gut erfassbar. Deshalb eignet sie sich unter den beschriebenen Veränderungen am meisten für physiologische Untersuchungen. Unter den zu untersuchenden Fragen erschien zunächst folgende besonders interessant: In welcher Beziehung stehen die Vorgänge, welche der Puppenhäutung vorausgehen, also insbesondere die Umfärbung, zu dem hormonalen Aktionssystem, welches Häutungen und Metamorphose steuert?

Dieses System besteht aus dem Gehirn, den Prothoraxdrüsen und den Corpora allata. Jede Häutung wird letzten Endes ausgelöst durch das Gehirn, dessen

VOL. 3 1959

neurosekretorische Zellen ein adenotropes Hormon produzieren, welches auf die Prothoraxdrüsen wirkt. Es veranlasst diese, ihrerseits ein Hormon in den Körper abzugeben. Dieses Hormon der Prothoraxdrüse ist das eigentliche Häutungshormon. Es löst in der Epidermis die Häutungsvorgänge aus. Bei alleiniger Wirkung dieses Hormons erfolgt die Häutung zur Imago, also die Metamorphose. In der jungen Larve aber geben gleichzeitig die Corpora allata ein anderes Hormon ab, welches eine vorzeitige Metamorphose verhindert und die Häutungen zu Larvenhäutungen, in schwächerer Konzentration zur Puppenhäutung umwandelt, das sog. Juvenilhormon. Von den erwähnten Hormonen konnte bisher das Häutungshormon der Prothoraxdrüse in kristallisierter Form isoliert werden durch BUTENANDT und KARLSON (1954). Den von ihnen isolierten Stoff nannten sie α-Ecdyson.

Bei Schmetterlingsraupen lässt sich die Puppenhäutung des hinteren Körperabschnitts verhindern durch eine rechtzeitig gelegte Ligatur, welche die Hormonzufuhr unterbindet. Die Tiere durchlaufen eine kritische Periode (k.P.), in der sie zur Puppenhäutung determiniert werden. Eine Schnürung nach der k.P. vermag deshalb die Häutung nicht mehr zu verhindern. Man muss deshalb annehmen, dass das Hormon, welches die Häutung auslöst, also das Ecdyson, in dieser k.P. in den Körper ausgeschüttet wird (CASPARI und PLAGGE, 1936; KÜHN and PIEPHO, 1936). Bei *Cerura vinula* fällt diese k.P. der Puppenhäutung zusammen mit dem Übergang in die Vorpuppe. Sie liegt also *erst etwa 5 Tage nach der Umfärbung*. Aber auch die Umfärbung selber liess sich durch solche Ligaturen verhindern bis zu einer k.P., nach der die Raupe zur Umfärbung determiniert ist. Diese k.P. der Umfärbung liegt *am letzten Tag vor Sichtbarwerden der Umfärbung*. Auf Grund dieser Ergebnisse wurde vermutet, dass auch die Umfärbung durch einen stofflichen Faktor ausgelöst wird, der sich vom Vorderkörper her ausbreitet und durch eine Ligatur daran verhindert werden kann.

Es ergeben sich verschieden Möglichkeiten für den Zusammenhang zwischen dem umfärbungsauslösenden Faktor und den bekannten Hormonen, welche Häutungen und Metamorphose steuern:

(1) Ein eigenes Umfärbungshormon, das nicht mit einem der erwähnten Hormone identisch ist, wird 6 Tage vor dem Häutungshormon in den Körper abgegeben und löst die Umfärbung aus. Diese Vorstellung stimmt überein mit einer Vermutung von BECKER (1941), der annahm, dass vor dem Häutungshormon schon ein anderes Hormon in den Körper abgegeben wird, das die Epidermis erst zur Reaktion auf das Häutungshormon befähigt und welches er deshalb "Aktivierungshormon" nannte.

(2) Das Gehirnhormon aktiviert nicht nur die Prothoraxdrüsen sondern es löst zugleich die Umfärbung aus. Diese Annahme würde verständlich machen, weshalb die Umfärbung schon vor der k.P. der Puppenhäutung erfolgen kann. Sie wird aber unwahrscheinlich durch Schnürungsexperimente, die zeigten, dass bei *Cerura vinula* auch das Gehirnhormon, welches die Puppenhäutung auslöst, erst lange nach der Umfärbung in den Körper abgegeben wird (BÜCKMANN, 1953).

(3) Das Umfärbungshormon ist mit dem Häutungshormon der Prothoraxdrüse, dem Ecdyson identisch. Dann bleibt zu erklären, wieso die Auslösung der

Umfärbung schon 5 Tage vor der k.P. der Puppenhäutung erfolgen kann, in der doch nach unseren bisherigen Vorstellungen das Häutungshormon erst in den Körper ausgeschüttet wird.

Diese Erklärung ist möglich durch zwei verschiedene Zusatzannahmen:

(3a) Das Häutungshormon wird gar nicht erst in der k.P. der Puppenhäutung ausgeschüttet, sondern ist schon vorher in niedriger, langsam steigender Konzentration im Körper vorhanden und erreicht während der k.P. die zur Auslösung der Häutung nötige Konzentration. Vorher schon löst es in geringerer Konzentration die Umfärbung aus. Eine solche Vorstellung wurde von PIEPHO (1942) begründet, um die von ihm bei *Galleria* gefundene steigende Determination der Epidermis zur Puppenhäutung im letzten Larvenstadium zu erklären.

(3b) Das Hormon ist vor der k.P. der Puppenhäutung schon in voller Konzentration im Körper vorhanden. Um die Epidermis zur Puppenhäutung zu determinieren, braucht es aber eine gewisse Einwirkungszeit, die erst in der k.P. erreicht wird. Vorher kann, da das Hormon vermutlich im Körper laufend inaktiviert wird und nachgeliefert werden muss, die Häutung durch eine Ligatur verhindert werden. Schon vor der k.P. der Puppenhäutung könnte es die Umfärbung auslösen. Nach dieser Vorstellung unterscheiden sich also Umfärbung und Puppenhäutung nicht dadurch, dass sie durch verschiedene Konzentration des Hormons sondern dadurch, dass sie durch verschieden lange Einwirkungsdauer desselben ausgelöst werden. Diese Vorstellung stammt von KARLSON (1955), der besonders auf die Bedeutung des Zeitfaktors der Hormonwirkung hinwies.

VOL. 3 1959

Die Aufgabe der vorliegenden Arbeit ist es, experimentell zu prüfen, durch welche inneren und äusseren Faktoren die Umfärbung von *Cerura vinula* ausgelöst wird. Dabei ist insbesondere die Frage berücksichtigt, in welcher Beziehung die auslösenden Faktoren zu dem hormonalen System stehen, das Häutungen und Metamorphose steuert.

Das Ergebnis ist der Beweis, dass die Umfärbung ähnlich wie die Häutungen ausgelöst wird über das Häutungshormon Ecdyson, d.h. dass die Hypothese 3 von den oben aufgestellten Möglichkeiten zutrifft, und zwar lässt sich die unter (3a) genannte Version am besten mit den experimentellen Ergebnissen vereinbaren.

Die Untersuchung wurde in 6 Schritten durchgeführt, von denen jeweils das Ergebnis des einen die Voraussetzung für die Durchführung des folgenden bildete:

(1) Beobachtung des normalen Verlaufs der Umfärbung unter den verwendeten Zuchtbedingungen, insbesondere Festlegung des Umfärbungszeitpunktes und seiner natürlichen Schwankungsbreite.

(2) Prüfung des Einflusses von Nahrungsaufnahme und Wachstum sowie ihrer vorzeitigen Beendigung auf den Eintritt der Umfärbung.

(3) Beweis der Auslösung der Umfärbung vom Vorderkörper her und Ermittlung der kritischen Periode, in der diese Auslösung erfolgt, durch Schnürungsexperimente.

(4) Ermittlung der Lage umfärbungsauslösender Zentren im Vorderkörper durch Schnürungsversuche mit doppelter Ligatur.

(5) Prüfung des Häutungshormons α-Ecdyson auf seine umfärbungsauslösende Wirkung.

(6) Beweis der vorzeitigen Auslösbarkeit der Umfärbung auch bei jungen Larven.

Einige der Ergebnisse wurden bereits in kurzen Mitteilungen bekanntgegeben: BÜCKMANN (1956), KARLSON und BÜCKMANN (1956).

(B) MATERIAL UND METHODIK

Die Raupen wurden aus Eiern gezogen, die von Züchtern aus verschiedenen Teilen Süddeutschlands geliefert worden waren. Zur Ermittlung der Schwankungsbreite in der Dauer des letzten Larvenstadiums bis zur Umfärbung wurden auch Zuchten verwendet, deren Eier aus England stammten. Alle Raupen wurden in Thermostaten bei 25° C gehalten, anfangs zu mehreren, nach der letzten Larvenhäutung einzeln in Petrischalen. Futter: Schwarzpappel (*Populus nigra* L.). Alle Raupen wurden täglich zweimal kontrolliert.

(C) EXPERIMENTELLE UNTERSUCHUNGEN

(1) *Der normale Verlauf des letzten Larvenstadiums und der normale Umfärbungszeitpunkt*

Begründung. Die Kenntnis dieser Daten war selbstverständliche Voraussetzung aller weiterer Untersuchungen. Der äussere Ablauf der Veränderungen vor der Puppenhäutung war schon früher beschrieben worden (BÜCKMANN, 1953). Die damaligen Zuchten konnten aber nicht streng temperaturkonstant gehalten werden und die einzelnen Zuchten hatten sich in der durchschnittlichen Dauer der einzelnen Entwicklungsabschnitte unterschieden.

Methodik. Zur Beobachtung des normalen Umfärbungszeitpunktes und -verlaufs wurde jeweils ein Teil der Raupen jeder Zucht verwendet. Sie wurden in der oben angegebenen Weise gehalten und alle 12 Stunden auf den Eintritt von Häutungen und den Wechsel der Färbungsstadien hin kontrolliert. Die Raupen der ersten Zucht wurden von der letzten Larvenhäutung an auch täglich gewogen, um den normalen Verlauf des Wachstums bis zum Eintritt der Umfärbung zu verfolgen.

Ergebnisse. Im ersten Teil des letzten Larvenstadiums von der letzten Larvenhäutung bis zur Umfärbung ist die Raupe grün (Abb. 1a). Sie frisst fast ununterbrochen. Von Beginn der Umfärbung ab gestatten die äusseren Veränderungen eine Alterseinteilung der Raupen in Färbungsstadien (BÜCKMANN, 1953). Sie beginnen bei der Umfärbung mit dem Färbungsstadium I. Den Zustand der grünen, fressenden Raupe kann man deshalb als Färbungsstadium O bezeichnen. Die Dauer dieses Stadiums in den verschiedenen Zuchten zeigt Tabelle 1, den Verlauf der Gewichtszunahme in diesem Zeitraum Tabelle 2.

Das Färbungsstadium O endet, wenn die Raupe aufhört zu fressen. Sie sitzt dann eine bis mehrere Stunden unbeweglich und man erkennt den Beginn der Umfärbung. Das Färbungsstadium I umfasst den gesamten Prozess der Umfärbung, der 8–12 Stunden dauert. Da während dieser Zeit sich die Farbe von Stunde zu Stunde ändert, ist eine Unterteilung des Stadiums möglich: Stadium Ia: Erstes Sichtbarwerden der Umfärbung. Beginnend

im dorsalen Bereich neben der braunen Rautenzeichnung verdunkelt sich das Hellgrün der Raupe zu Olivgrün. Die Raupe sitzt währenddessen meist unbeweglich. Stadium Ib: Die rote Farbe der Epidermis wird deutlich. Die Raupe gibt ihren letzten Kotballen ab, der durch Exkreptigmente rot gefärbt ist, und beginnt ruhelos umherzukriechen. Stadium Ic: Während die Raupe mit dem Kokonbau beginnt, färbt sie sich immer dunkler und wird vorübergehend fast schwarz. All diese Veränderungen beruhen darauf, dass sich die Epidermis immer dunkler rot färbt, sodass schliesslich im Zusammenwirken mit der durchschimmernden grünen Körperflüssigkeit die schmutzige sehr dunkle Farbe des Stadiums Ic resultiert. Danach beginnt sich auch der Fettkörper unter der Epidermis rot zu färben. Dadurch hellt sich die Körperfarbe auf zu einem reinen Rot: Damit befindet sich die Raupe im Färbungsstadium II. Stadium III: Der Thorax färbt sich wieder grün. Das beruht auf dem Verblassen der roten Farbe der Epidermis. Das Abdomen bleibt weiterhin durch den durchschimmerden Fettkörper rot gefärbt. Stadium IV: Die weisse Rautenumrandung verblasst, die Raupe färbt sich einheitlich gelbbraun und nimmt die Vorpuppenform an (Abb. 1d). Gleichzeitig setzen in der Epidermis die Häutungsprozesse ein. Die gleichmässige Farbe der Vorpuppe wird wahrscheinlich bewirkt durch die Exuvialflüssigkeit welche sich zwischen der alten Cuticula und den ersten Schichten der neuen bildet, sodass der rote Fettkörper nicht mehr durchschimmert. Vier bis fünf Tage nach dem Beginn des Stadiums IV häutet sich die Vorpuppe zur Puppe.

VOL. 3 1959

TABELLE 1—DAUER DES LETZTEN LARVENSTADIUMS BIS ZUR UMFÄRBUNG (IN TAGEN)

Herkunft des Materials	Zahl d. Tiere	Höchster u. niedrigster beobachteter Wert	Mittelwert	Standart-abweichung	Bemer-kungen
Frankfurt	10	6–8	$6{,}6 \pm 0{,}2$	0,7	täglich gewogen
Wasserburg/Inn	36	5–6½	$5{,}62 \pm 0{,}05$	0,7	—
Kent, England	24	5–7	$5{,}6 \pm 0{,}1$	0,5	—
Gesamte ungestörte Tiere	60	5–7	$5{,}62 \pm 0{,}05$	0,4	—

Besprechung. Die Werte für die Dauer des Stadiums O (letzte Larvenhäutung bis Umfärbung) stimmen in den verschiedenen Zuchten gut überein. Eine Abweichung zeigen nur die Tiere, die täglich gewogen wurden. Bei ihnen trat die Umfärbung durchschnittlich einen ganzen Tag später ein. Diese Verzögerung hängt sicherlich mit der täglichen Störung zusammen. Die Tiere mussten zur Wägung immer gewaltsam von ihren Blättern gelöst werden. Möglicherweise brauchten sie nach jeder Wägung längere Zeit, bis sie wieder ihre typische Fressstellung auf dem Rande der Blätter gefunden hatten und weiterfrassen. Jedenfalls zeigten die anderen Tiere derselben Zucht, die zu Schnürungsversuchen verwendet wurden, genau den gleichen Wert für die Dauer des Stadiums O wie die übrigen Zuchten, nämlich im Mittel 5, 6 Tage. Dieser Wert kann daher als die normale Dauer von der letzten Larvenhäutung bis zur Umfärbung bei gesunden ungestörten Tieren und den angewendeten Zuchtbedingungen (25° C) gelten.

(2) *Der Einfluss von Nahrungsaufnahme und Wachstum auf den Umfärbungszeitpunkt*

Begründung. Neben der Temperatur ist es an äusseren Faktoren vor allem die Ernährung, die den Zeitpunkt der Umfärbung beeinflussen könnte. Es ist denkbar, dass der Eintritt der Umfärbung ausgelöst wird durch das Ende der Nahrungsaufnahme oder dadurch, dass die Raupe eine gewisse Grösse erreicht hat. In beiden Fällen würde die Ernährung als "Zeitgeber" dienen für die Auslösung der Umfärbung. Ein ähnlicher Zusammenhang konnte bei der Wanze *Rhodnius prolixus* nachgewiesen werden (WIGGLESWORTH, 1943). Hier ist es die Streckung des Abdomens durch eine grosse Blutmahlzeit, welche das hormonale Aktionssystem zur Auslösung der Häutung veranlasst. Die Frage nach dem Einfluss der Ernährung auf den Eintritt der Umfärbung musste als allererste geklärt werden, weil praktisch jeder experimentelle Eingriff wie Schnürungen und Operationen vor der Umfärbung eine vorzeitige Beendigung der Nahrungsaufnahme und damit des Wachstums bedingt. Dadurch könnte der Eintritt der Umfärbung beschleunigt oder verhindert und so das Ergebnis des Experiments verfälscht werden.

Methodik. Der Verlauf des Wachstums im letzten Larvenstadium bis zur Umfärbung wurde verfolgt bei einer Gruppe von Raupen, die täglich gewogen wurden. Da sich zeigte, dass durch diese tägliche Störung die Entwicklung beeinträchtigt wurde (s. Abschnitt 1), wurde bei weiteren Versuchsgruppen nur das Umfärbungsgewicht, das Körpergewicht zu Beginn der Umfärbung, ermittelt. Der Einfluss einer vorzeitigen Beendigung der Nahrungsaufnahme auf die Umfärbung wurde geprüft an Tieren, denen verschieden lange nach der letzten Larvenhäutung das Futter entzogen wurde. Ihr Körpergewicht zur Zeit des Futterentzugs wurde gewogen und der Eintritt der Umfärbung beobachtet.

TABELLE 2—VERLAUF DER GEWICHTSZUNAHME VON DER LETZTEN LARVENHÄUTUNG BIS ZUR UMFÄRBUNG

Zeit (Tage nach der letzten Larvenhäutung)	Zahl d. Tiere	Kleinster und grösster Wert g	Mittelwert g	Standartabweichung g	Bemerkungen
0	10	0,4—0,9	0,7		
1	10	0,7—1,5	1,1		
2	10	1,2—3,0	2,2		
3	10	1,9—3,4	2,8		
4	10	2,6—4,2	3,4		
5	10	3,0—5,0	4,1		
6	10	3,4—4,9	4,0		5 der Tiere schon umgefärbt
Umfärbungsgewicht der täglich gewogenen	10	3,1—4,8	4,1 ±0,2	0,6	
Umfärbungsgewicht ungestörter Raupen	24	3,7—5,6	4,5 ±0,1	0,4	

Bild 1(a)

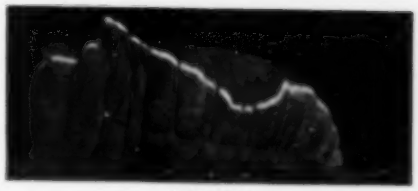


Bild 1(b)

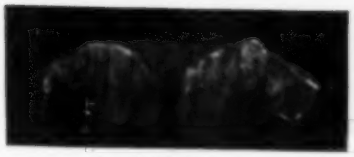


Bild 1(c)

Bild 1(d)

Bild 1(e)

BILD 1. Die Färbungsstadien von *Cerura vinula*. (a) Stadium O (grüne, fressende Raupe im letzten Larvenstadium). (b) Stadium II (Rotfärbung nach dem Verlassen des Futters). (c) Stadium III (Wiederentfärbung der Epidermis im Kokon). (d) Stadium IV (Vorpuppe). (e) Puppe, dorsal geöffnet, um die rote Farbe des Fettkörpers zu zeigen.

Bild 2(a)

Bild 2(b)

BILD 2. Ergebnisse von Schnürungsexperimenten. (a) Vor der kritischen Periode der Umfärbung geschnürte Raupe: Vorderstück Stadium II, Hinterstück Stadium O. (b) Nach der kritischen Periode der Umfärbung geschnürte Raupe. Beide Körperstücke haben sich rot umgefärbt, und das Stadium II erreicht. Das Hinterstück ist auf Stadium II stehengeblieben, das Vorderstück hat sich weiterentwickelt und das Stadium III erreicht.

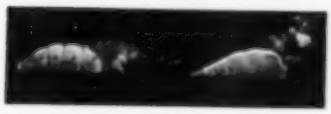


Bild 3

BILD 3. Ergebnisse von Injektionsexperimenten. Testabdomina: links nach Injektion von 3300 Calliphora-Einheiten Ecdyson—man erkennt die Vorpuppenform und die Rotfärbung des Fettkörpers, der im dorsalen Rereich durchschimmert—rechts nach Injektion von Ringerlösung zum Vergleich.

Bild 4

BILD 4. Durch Dekapitation vorzeitig bei einer Raupe des vorletzten Larvenstadiums ausgelöste Umfärbung (links). Zum Vergleich eine grüne fressende Raupe des letzten Stadiums, an dessen Ende die Umfärbung normalerweise erfolgt (rechts).

VOL.
3
1959

Ergebnisse. Den Verlauf der Gewichtszunahme im letzten Stadium zeigt Tabelle 2. An den beiden ersten Tagen verdoppelt sich das Gewicht etwa täglich. Dann verlangsamt sich die Gewichtszunahme merklich. Vier Tage nach der letzten Larvenhäutung haben fast alle Tiere ein Körpergewicht von >3g erreicht. Das höchste Gewicht wurde bei den meisten Tieren schon bei der letzten Wägung vor der Umfärbung gemessen. Bei Deutlichwerden der Umfärbung hatten sie schon wieder an Gewicht verloren, weil sie zu diesem Zeitpunkt schon einige Stunden nicht mehr gefressen und den Darm entleert haben. Das Umfärbungsgewicht der ungestörten Tiere lag wenig höher als das der täglich gewogenen. Das gemeinsame Ergebnis beider Versuchsserien ist, dass das normale Umfärbungsgewicht im Mittel etwas über 4g liegt.

TABELLE 3—WIRKUNG VORZEITIGEN NAHRUNGSENTZUGS

Zeitpunkt des Nahrungsentzugs (Tage nach d. letzten Häutung)	Zahl d. Tiere	Durchschnittsgewicht g	Umgefärbt	Zeitpunkt d. Umfärbung (Tage n. d. letzten Häutung)
2–2½	14	2,8 ± 0,1	4 = 29%	6½–10½ M = 9,0 ± 0,9
3–3½	14	3,8 ± 0,3	10 = 71%	5½–10½ M = 6,8 ± 0,5
4–4½	16	3,2 ± 0,2	14 = 88%	4½–8½ M = 5,7 ± 0,4

Die Wirkung vorzeitigen Nahrungsentzugs zeigt Tabelle 3. Früher Nahrungsentzug, 2–2½ Tage nach der letzten Häutung, hat einen deutlichen Einfluss auf die Umfärbung: Bei mehr als ⅔ der Tiere unterblieb die Umfärbung ganz. Die Tiere starben nach längerer Zeit ohne Umfärbung und Verpuppung. Bei den übrigen Tieren erfolgte die Umfärbung merklich verspätet, durchschnittlich um >4 Tage später als bei normalen Tieren. In vielen Fällen verlief sie nicht normal sondern nur schwach und undeutlich. Auch Nahrungsentzug 3–3½ Tage nach der letzten Häutung beeinträchtigte die Umfärbung noch merklich. Sie unterblieb noch bei 29% der Tiere ganz und war bei den übrigen gegenüber normalen Tieren um mehr als einen Tag verspätet, obwohl Tiere verwendet worden waren, die zur Zeit des Nahrungsentzuges schon hohe Körpergewichte von 3g erreicht hatten. Nahrungsentzug 4–4½ Tage nach der letzten Häutung beeinträchtigte dagegen die Umfärbung nicht mehr merklich. Mit nur 2 Ausnahmen färbten sich alle Versuchstiere im normalen Ausmass um, und zwar etwa wie Normaltiere im Mittel 5, 7 Tage nach der letzten Larvenhäutung. Die Umfärbung erfolgte also zum normalen Zeitpunkt, obgleich die Versuchstiere nur ¾ des normalen Umfärbungsgewichtes erreicht hatten.

Besprechung. Offensichtlich ist eine Periode der Nahrungsaufnahme von etwa 4 Tagen zu Beginn des letzten Larvenstadiums erforderlich, um die Tiere zu

einer normalen Umfärbung in die Lage zu versetzen. In dieser Zeit wird ein Gewicht von >3g erreicht. Nach Abschluss dieser obligatorischen Fressperiode wird die Umfärbung von Nahrungsaufnahme und Wachstum unabhängig. Sie erfolgt—gleich ob die Raupe vorzeitig zu fressen aufgehört hat oder bis zum normalen Umfärbungsgewicht weitergewachsen ist—zum normalen Zeitpunkt, im Mittel ca. 5, 6 Tage nach der letzten Larvenhäutung.

Die Ergebnisse zeigen, dass durch vorzeitige Unterbrechung der Nahrungsaufnahme keinesfalls eine vorzeitige Auslösung der Umfärbung bewirkt wird. Wird der Zeitpunkt der Umfärbung durch sehr frühen Nahrungsentzug überhaupt beeinflusst, dann im Gegenteil im Sinne einer Verzögerung der Umfärbung. Andererseits ist zur Auslösung der Umfärbung auch nicht das Erreichen des normalen Umfärbungsgewichtes erforderlich, denn die Tiere, die 4–4½ Tage nach der letzten Häutung vom Futter genommen worden waren, hatten nur ¾ dieses Gewichtes erreicht und sich dennoch zum normalen Zeitpunkt umgefärbt.

Die Ergebnisse dieses Abschnittes werden bestätigt durch die Ergebnisse späterer Versuchsreihen. Zu den Schnürungsversuchen(Abschnitt 3) wurden Tiere verwendet, die 3½–4½ Tage nach der letzten Häutung vom Futter genommen wurden. 96 Prozent von ihnen hatten zu dieser Zeit ein Gewicht von >3g erreicht. Die durch die Schnürung nicht beeinflusste Umfärbung der Vorderstücke erfolgte in allen 181 Fällen normal und zum normalen Zeitpunkt, nämlich im Mittel 5, 6 Tage nach der letzten Larvenhäutung. (s.S. 169).

(3) *Die Auslösung der Umfärbung vom Vorderkörper her*

Begründung. In den früheren Untersuchungen war es gelungen, durch eine Ligatur, welche den Körper in zwei Stücke trennte, die Umfärbung des hinteren Körperstückes zu verhindern. Es zeigte sich auch, dass es eine kritische Periode gibt, nach welcher das Hinterstück zur Umfärbung determiniert ist und durch eine Ligatur nicht mehr daran verhindert werden kann. Es wurde vermutet, dass die Umfärbung während dieser k.P. von einem im Vorderkörper gelegenen Zentrum her ausgelöst wird. Dieser Schluss wäre bewiesen, wenn sich gezeigt hätte, dass nach der Trennung das Hinterstück nicht mehr zur Umfärbung in der Lage ist, wohl aber das Vorderstück. Dieser Beweis fehlte, weil es nicht gelang, die Vorderstücke der durchschnürten Tiere am Leben zu halten. Die im Folgenden beschriebenen Versuche zeigen, dass tatsächlich im Vorderkörper ein umfärbungsauslösendes Zentrum vorhanden sein muss, welches im Abdomen fehlt. Zugleich konnte die k.P., in der die Auslösung der Umfärbung erfolgt, genauer ermittelt werden.

Methodik. Die Versuchstiere wurden 3½–4½ Tage nach der letzten Larvenhäutung vom Futter genommen und erst 12 Stunden später mittels einer Ligatur im ersten Abdominalsegment durchschnürt. In Vorversuchen hatte sich gezeigt, dass bei solchen, nach 12 stündiger Hungerzeit geschnürten Raupen auch fast alle Vorderstücke am Leben bleiben.

Ergebnisse. Von 205 in dieser Weise geschnürten Raupen überlebten bei 181 die Vorderstücke bis nach ihrer Umfärbung, 2 sogar bis nach der Puppenhäutung.

Alle 181 Vorderstücke färbten sich im normalen Ausmass und zum normalen Zeitpunkt um wie die ungestörten Kontrolltiere, nämlich im Mittel $5{,}67 \pm 0{,}05$ Tage nach der letzten Larvenhäutung. Von den zugehörigen Hinterstücken starb 1 sofort nach der Schnürung. Von den übrigen 180 Hinterstücken erfolgte bei 10 eine vollständige Umfärbung gleichzeitig mit derjenigen der zugehörigen Vorderstücke. Weitere 10 färbten sich nicht vollständig um, sondern nur im dorsalen Bereich. Diese Erscheinung kann als *Teilumfärbung* bezeichnet werden, entsprechend der von KÜHN und PIEPHO (1936) bei Schnürungsexperimenten beobachteten Teilverpuppung. Bei 3 der teilumgefärbten Hinterstücke war ausserdem der Eintritt der Umfärbung gegenüber der des zugehörigen Vorderstückes um $\frac{1}{2}$ Tag verzögert. Die umgefärbten Hinterstücke blieben in ihrer Entwicklung auf dem Färbungsstadium II stehen, während sich die Vorderstücke weiterentwickelten, solange sie am Leben blieben, und die Stadien III und IV, ja, wie erwähnt, in 2 Fällen sogar die Puppenhäutung erreichten. 160 Hinterstücke blieben grüne Dauerraupen. (Abb. 2).

TABELLE 4—ERGEBNISSE VON SCHNÜRUNGSEXPERIMENTEN, EINE LIGATUR IM ERSTEN ABDOMINALSEGMENT

Zeitraum Schnürung bis Umfärbung des Vorderstücks Tage	Zahl d. Tiere	Davon Hinterstücke voll umgefärbt	Hinterstücke teilumgefärbt	Insgesamt umgefärbte Hinterstücke
0–$\frac{1}{2}$	36	10	8	18 = 50%
$\frac{1}{2}$–1	77	—	2	2 = 3%
>1	67	—	—	0%

Die Ergebnisse sind zusammengefasst in Tabelle 4, aus der die Lage der k.P. deutlich hervorgeht. Es zeigt sich nämlich, dass es vom Schnürungszeitpunkt abhängt, ob das Hinterstück grün bleibt, oder ob es sich mit dem Vorderstück zusammen umfärbt. Von den innerhalb 12 Stunden vor der Umfärbung geschnürten Tieren vermochte sich genau die Hälfte der Hinterstücke umzufärben. War die Schnürung mehr als 1 Tag vor der Umfärbung erfolgt, so wurde die Umfärbung des Hinterstückes auf jeden Fall verhindert.

Von den 160 grüngebliebenen Hinterstücken wurden 40 zur Kontrolle bis zu ihrem Tod unbehandelt gelassen. 36 weitere erhielten Injektionen von Insektenringerlösung. Diese insgesamt 76 Kontrolltiere überlebten die Umfärbung der zugehörigen Vorderstücke um bis zu 27 Tage, im Mittel um 8, 9 Tage, ohne irgendwelche Anzeichen von Umfärbung zu zeigen. Die restlichen grünen Hinterstücke wurden zu anderen Experimenten verwendet (Abschnitt 5).

Besprechung. Durch die Ligatur im ersten Abdominalsegment wurden die Tiere geteilt in ein Vorderstück, bestehend aus Kopf, Thorax und einem Teil des

ersten Abdomensegmentes und ein Hinterstück, bestehend aus dem Rest des Abdomens. Wenn man von dem vorderen Teil des ersten Abdominalsegmentes einmal absehen darf, erfolgte die Trennung also etwa auf der Thorax-Abdomen-Grenze. Das Vorderstück: Kopf+Thorax ist auf jeden Fall zur Umfärbung in der Lage, das Abdomen nur in einem kleinen Teil der Fälle, wenn die Schnürung sehr spät vor der Umfärbung erfolgt war. Der Vorderkörper muss also ein umfärbungsauslösendes Zentrum enthalten, welches dem Abdomen fehlt. Ob dieses im Kopf oder im Thorax liegt, lässt sich nach den beschriebenen Versuchen noch nicht entscheiden. Jedenfalls wird von dort die Umfärbung des ganzen Körpers ausgelöst, sodass sie in einem rechtzeitig davon getrennten Abdomen unterbleibt. Da von den innerhalb von 12 Stunden nach der Schnürung umgefärbten Tieren noch bei genau 50 Prozent auch die Hinterstücke sich umfärbten, ergibt sich, dass die kritische Periode, in der die Umfärbung vom Vorderkörper her ausgelöst wird, im Durchschnitt etwa 6 Stunden vor der Umfärbung durchlaufen wird.

Besonders interessant ist das Auftreten von Teilumfärbungen. Die betreffenden Tiere sind offensichtlich genau während der kritischen Periode geschnürt worden und hatten den Anstoss zur Umfärbung noch zum Teil erhalten, aber nicht mehr im vollen Ausmass. Die Auslösung der Umfärbung gehorcht also keinem Alles-oder-nichts-Gesetz. Die Ergebnisse sprechen dafür, dass die Auslösung durch einen stofflichen Faktor geschieht, der vom umfärbungsauslösenden Zentrum während der kritischen Periode der Umfärbung abgegeben wird, und der im Falle der Teilumfärbungen im abgeschnürten Abdomen noch nicht die volle Konzentration erreicht hatte.

Die Tatsache, dass die Vorderstücke am Leben bleiben, wenn die Schnürung nach 12-stündiger Hungerzeit erfolgt, spricht für die Richtigkeit der Vermutung von CASPARI und PLAGGE (1935), dass die hohe Sterblichkeit der Vorderstücke von durchschnürten Raupen darauf zurückzuführen ist, dass sich der Darm nicht entleeren kann.

Die Tatsache, dass die umgefärbten Hinterstücke in ihrer Entwicklung auf dem Färbungsstadium II stehenblieben, während sich die Vorderstücke weiterentwickelten über die Stadien III und IV zur Puppenhäutung, spricht dafür, dass auch die weiteren Entwicklungsschritte zwischen der Umfärbung und der Puppenhäutung vom Vorderkörper her ausgelöst werden müssen.

(4) *Die Lage der umfärbungsauslösenden Zentren*

Begründung. Die Frage, ob das umfärbungsaulösende Zentrum im Kopf oder im Thorax gelegen ist, konnte untersucht werden mit Hilfe doppelter Schnürung, wobei eine Ligatur, wie in den bisherigen Versuchen, Thorax und Abdomen voneinander trennt, eine zweite Kopf und Thorax. Bei dieser Anordnung muss sich die Lage des gesuchten Zentrums in folgender Weise ergeben: Liegt das Zentrum im Thorax, dann müssen sich bei allen Tieren die Vorderstücke umfärben, die dies Zentrum ja noch enthalten, darüber hinaus bei den nach der k.P. geschnürten auch die Hinterstücke. Es darf also nur zwei Gruppen von

Versuchstieren geben: Abb. 5(a) und (b). Liegt dagegen das Zentrum im Kopf, so müssen sich Thorax und Abdomen, da sie gleichermassen vom Kopf getrennt sind, stets gleichartig verhalten. Entweder sie bleiben beide grün, oder, falls nach der k.P. erst geschnürt worden war, sie färben sich beide um. Es wäre allerdings in diesem Falle eine Verfälschung der Ergebnisse möglich durch eine langsame Ausbreitung des umfärbungsauslösenden Faktors vom Kopfe her: Zur Zeit der Schnürung könnte dieser Faktor den Thorax bereits in ausreichender Konzentration erreicht haben, das Abdomen aber noch nicht, sodass doch eine Umfärbung nur des Vorderstücks resultieren würde. Diese Fehlerquelle liess sich aber leicht ausschalten, indem die vordere Ligatur 6 Stunden früher gelegt wurde als die hintere, sodass ein etwa teilweise ausgeschütteter umfärbungsauslösender Faktor Zeit genug hatte, sich in Thorax und Abdomen gleichmässig zu verteilen. Im Falle der Lage des umfärbungsauslösenden Zentrums im Kopfe, darf es also wiederum nur 2 Gruppen von Versuchstieren geben: Abb. 5 (a) und (c): ganz umgefärbte und gar nicht umgefärbte Tiere.

In einer zweiten Versuchsserie wurde der Kopf vom Thorax nicht durch eine Ligatur getrennt sondern stattdessen das Gehirn herausgenommen, um die Bedeutung speziell des Gehirns für die Auslösung der Umfärbung zu prüfen.

Methodik.—Serie A: 37 Raupen wurden 4 Tage nach der letzten Larvenhäutung vom Futter genommen. 6 Stunden später wurde ihnen durch eine Ligatur der Kopf vom Thorax getrennt und nach weiteren 6 Stunden durch eine zweite Ligatur im ersten Abdominalsegment der Thorax vom Abdomen getrennt.

Serie B: 17 weiteren Raupen wurde 4 Tage nach der letzten Häutung das Gehirn exstirpiert (Methodik nach KÜHN und PIEPHO, 1936). 6 Stunden darauf wurde ihnen ebenfalls durch eine Ligatur im ersten Abdominalsegment der Thorax vom Abdomen getrennt.

TABELLE 5—ERGEBNISSE DOPPELTER SCHNÜRUNGSEXPERIMENTE
(A) 1 LIGATUR HINTER DEM KOPF, 1 LIGATUR IM ENSTEN ABDOMINALSEGMENT
(B) ENTHIRNUNG UND EINE LIGATUR IM ENSTEN ABDOMINALSEGMENT

Zeitraum Schnürung bis Umfärbung d. Vorderstücks Tage	beide Körperstücke umgefärbt			nur Vorderstück umgefärbt			beide Stücke nicht umgefärbt		
	A	B	zus.	A	B	zus.	A	B	zus.
0–½	2	1	**3**	—	—	—			
½–1	2	—	**2**	1	3	**4**			
1	—	—	—	6	4	**10**			
							25	9	**34**

Ergebnisse (*Tab.* 5). Bei dem grössten Teil der Tiere beider Versuchsserien blieb die Umfärbung sowohl im Thorax als auch im Abdomen aus. Die Abdomina blieben als grüne Dauerraupen noch lange ohne irgendwelche Spuren von Um-

färbung am Leben, und zwar in Serie A bis zu 22½ Tage, im Mittel 11,5 Tage nach der letzten Larvenhäutung, in Serie B bis 19, im Mittel 9,4 Tage nach der letzten Larvenhäutung. Die Vorderstücke überlebten als grüne Dauerraupen in Serie A bis 12, im Mittel 7,5, in Serie B bis 7, im Mittel 6,5 Tage nach der letzten Larvenhäutung ebenfalls ohne jede Spur von Umfärbung.

Bei einem kleinen Teil der Tiere in beiden Serien färbten sich beide Körperstücke um, und zwar in diesen Fällen immer weniger als 1 Tag nach der Schnürung. Neben diesen beiden Gruppen gab es in beiden Serien noch eine dritte: Bei diesen Tieren färbte sich nur der Thorax um. Die Hinterstücke überlebten als grüne Dauerraupen die Umfärbung der zugehörigen Vorderstücke in Serie A bis zu 15½ Tage, im Mittel 9,1 Tage, in Serie B bis zu 2, im Mittel 1,7 Tage. Die Umfärbung des Thorax erfolgte in dieser Gruppe ½ bis 4½ Tage nach der Schnürung.

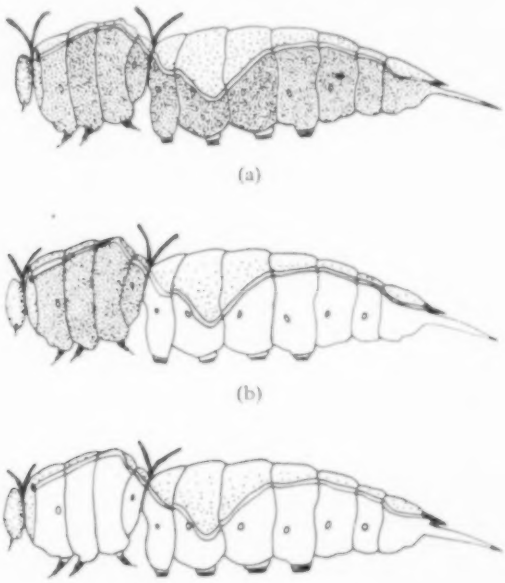


ABBILDUNG 5. Doppelt geschnürte Raupen. (a) Beide Stücke umgefärbt, Umfärbung innerhalb 12 Stunden nach der Schnürung. (b) Nur das Vorderstück umgefärbt, Umfärbung 1–4 Tage nach der Schnürung. (c) Beide Stücke nicht umgefärbt.

Besprechung. Die Ergebnisse beider Versuchsserien gleichen sich. Enthirnung wirkt also wie Abschnürung des ganzen Kopfes. Das Ergebnis ist aber weder das für eine Lage des Zentrums im Kopf, noch das für seine Lage im Thorax vorhergesagte. Es gibt nicht—wie in jedem der beiden Fälle zu erwarten—nur 2 sondern 3 Gruppen von verschieden reagierenden Versuchstieren.

In Fällen wo nach der k.P. der Umfärbung geschnürt worden war, innerhalb eines halben Tages vor der Umfärbung also, färbten sich beide Körperstücke um, wie in jedem Falle zu erwarten. Bei den meisten Tieren vermochte die Trennung vom Kopf bzw, vom Gehirn die Umfärbung beider Körperstücke zu verhindern. Es sind also im Kopf gelegene Organe, sehr wahrscheinlich das Gehirn zur Auslösung der Umfärbung notwendig. Obwohl sie aber gleichermassen vom Gehirn getrennt waren, verhielten sich Thorax und Abdomen nicht immer gleich. Das Abdomen färbte sich, wie in allen bisherigen Versuchen, entweder innerhalb eines Tages oder gar nicht um. Der Thorax dagegen vermochte sich in manchen Fällen noch mehrere Tage nach seiner Trennung vom Kopf bzw, dem Gehirn umzufärben. Das Gehirn wird also schon mehr als 1 Tag vor der Umfärbung für deren Auslösung entbehrlich, und das umfärbungsauslösende Zentrum, welches weniger als $\frac{1}{2}$ Tag vor der Umfärbung wirkt, ist im Thorax noch vorhanden.

Die Ergebnisse müssen daher folgendermassen gedeutet werden: Ein im Kopf gelegenes Zentrum, ziemlich sicher das Gehirn, gibt den ersten Anstoss zur Umfärbung während einer ersten k.P. mehr als 1 Tag vor der Umfärbung. Es determiniert nicht direkt die Rumpfepidermis zur Umfärbung sondern wirkt auf ein zweites, im Thorax gelegenes Zentrum. Dieses bewirkt daraufhin in einer zweiten k.P., der eigentlichen k.P. der Umfärbung, die Determination der Rumpfepidermis zur Umfärbung. Vermutlich besteht die Wirksamkeit dieses thorakalen Zentrums in der Abgabe eines umfärbungsauslösenden Stoffes.

Sicherlich darf aus dem Verhalten einiger Vorderstücke nicht der Schluss gezogen werden, dass die erste k.P. in der die Auslösung vom Kopfe her erfolgt, schon 4 Tage vor der Umfärbung liegt. Es kann vielmehr angenommen werden, dass bei diesen Tieren die vordere Schnürung bzw. Enthirnung genau während der ersten k.P. erfolgt war, sodass das thorakale Zentrum den Anstoss zur Auslösung der Umfärbung nicht mehr in vollem Umfange erhielt, wodurch eine Verzögerung der Auslösung zustandekam.

(5) *Das Häustungshormon Ecdyson als umfärbungsauslösender Faktor*

Begründung. Die bisherigen Versuche zeigen, dass die Umfärbung ausgelöst wird von einem thorakalen Zentrum, wahrscheinlich durch die Abgabe eines umfärbungsauslösenden Stoffes. Es ist durchaus denkbar, dass dieses thorakale Zentrum die Prothoraxdrüse ist, und dann könnte der umfärbungsauslösende Faktor mit ihrem Häutungshormon, dem Ecdyson, identisch sein. Die Ergebnisse der Schnürungsexperimente lieferten zugleich die Möglichkeit, diese Vermutung experimentell zu prüfen. Es war gelungen (Abschnitt 3), Tiere zu erhalten, deren Thorax sich umgefärbt hatte, während das Abdomen grün blieb. Die Tiere sind kompetent zur Umfärbung, wie das Schicksal des Vorderstückes zeigt. Dem Abdomen fehlt dazu nur der umfärbungsauslösende Faktor, dessen Eindringen durch die Ligatur verhindert ist. Das Abdomen müsste also durch Zuführung des fehlenden Umfärbungsfaktors zur Umfärbung gebracht werden können.

VOL. 3 1959

Solche grüngebliebenen Abdomina eignen sich also, um Organe oder Stoffe auf ihren Gehalt an Umfärbungsfaktor hin zu prüfen.

Durch das freundliche Entgegenkommen von Herrn Dr. Karlson, München, wurde es möglich, einen hockwirksamen Ecdyson-haltigen Extrakt und eine Lösung des reinen kristallisierten α-Ecdysons an solchen grünen Testabdomina zu prüfen.

Methodik. 72 grüne, durch Abschnürung isolierte Testabdomina, deren zugehörige Vorderstücke sich umgefärbt hatten, erhielten Injektionen von Hormon oder hormonhaltigem Extrakt, 41 weitere erhielten als Kontrolltiere Injektionen der reinen Lösungsmittel; Insektenringer oder Wasser. Die Injektionen wurden frühestens ½ Tag nach der Umfärbung der zugehörigen Vorderstücke vorgenommen. Sie geschah mittels einer Mikroinjektionsspritze nach BERGOLD (1941). Zur Injektion musste die sehr dicke und zähe Cuticula der Raupen durch einen Schnitt geöffnet werden. Der Verschluss auch kleiner Wunden macht bei diesen Raupen Schwierigkeiten, da er durch Kontraktionen der gesamten Rumpfmuskulatur immer wieder aufgesprengt wird. Diese Schwierigkeit wurde auf folgende Weise umgangen: Der Einschnitt geschah im vordersten Segment des Testabdomens, und die Kanüle wurde bis ins zweite Segment eingeführt. Dann wurde eine neue Ligatur auf der Grenze zwischen dem vordersten und dem zweiten Segment gelegt und angezogen. Nach der Injektion wurde dann durch Zuziehen dieser Ligatur das vorderste Segment völlig abgeschnürt und so das Testabdomen um 1 Segment verkürzt.

Injiziert wurden reines Kristallisat von α-Ecdyson, gelöst in Wasser, und ein hochgereinigtes Konzentrat eines Ecdyson-haltigen Extraktes, gelöst in Insektenringerlösung. Die Wirksamkeit des Konzentrats war von KARLSON vorher im *Calliphora*-Test ermittelt worden. Die verwendete Stammlösung enthielt 133 *Calliphora*—Einheiten (CE) pro mm^3. Eine CE entspricht 0,0075γ des reinen kristallisierten Ecdysons. Es ist diejenige Hormonmenge, die pro Tier injiziert werden muss, um bei abgeschnürten *Calliphora*-Larven in 50–70% der Fälle Pupariumbildung auszulösen (KARLSON und HANSER 1952). Die verwendeten Dosen wurden in 10–25 mm^3 Flüssigkeitsmenge gelöst injiziert. 50 grüne Testabdomina erhielten Injektionen von Hormonkonzentrat. Die Höhe der Dosen betrug 13; 66; 330; 3300 und 6600 CE. 36 Testabdomina erhielten zur Kontrolle reine Ringerlösung. Reines, kristallisiertes Hormon konnte der geringen zur Verfügung stehenden Menge wegen nur in kleinen Dosen verwendet werden. 22 Testabdomina erhielten Dosen von 13; 66 und 130 CE, 5 weitere erhielten zur Kontrolle entsprechende Flüssigkeitsmengen reinen Wassers injiziert.

Um die durch die Injektionen bewirkte Entwicklung möglichst vollständig zu erfassen, wurde die Mehrzahl der Testabdomina beobachtet, bis Bewegungslosigkeit und Aussetzen des Herzschlags den bevorstehenden Tod anzeigten. Das war spätestens 13½ Tage nach der Injektion der Fall. Dann wurden sie unter 0,9%iger NaCl-Lösung seziert, um den Färbungszustand der inneren Organe zu untersuchen. 10 der Testabdomina wurden schon früher lebend nach BOUIN fixiert zur Untersuchung ihres histologischen Zustands.

Ergebnisse. Alle Testabdomina, die Injektionen von 330 CE und mehr erhalten hatten, und ein Teil der Tiere mit kleineren Dosen zeigten deutliche Reaktionen auf das Hormon, wie sie nach Injektionen von Wasser oder Ringerlösung in keinem Falle beobachtet wurden. Die Zeit von der Injektion bis zum Sichtbarwerden der Reaktion betrug 1–3 Tage. Deswegen kann nur bei solchen Tieren ein negativer Versuchsausfall registriert werden, die länger als 3 Tage nach der Injektion am Leben blieben, ohne eine Reaktion zu zeigen. In jedem Falle blieben die Tiere in dem innerhalb von 3 Tagen erreichten Zustand stehen. Spätere Reaktionen wurden nicht beobachtet.

Die äussere Beobachtung und die Sektionen der Versuchstiere ergaben für die Versuche mit Hormonkonzentrat folgendes:

13 CE: Von 5 Tieren reagierten 3. Zwei Tage nach der Injektion färbte sich bei ihnen der grüne Epidermisbereich dorsal neben der Rautenzeichnung schwach aber deutlich rosa. Schon am lebenden Tier war zu erkennen, dass die rötliche Färbung in der Epidermis lokalisiert war. (Die Färbung der Epidermis lässt sich am lebenden Tier an Epidermisfalten erkennen, dort wo die Epidermis doppelt liegt, ohne dass sich Fettgewebe zwischen den beiden Schichten befindet, wie dies an den hinteren Segmenträndern der Fall ist, oder durch das Zusammenfassen der Epidermis mit einer Pinzette leicht erreicht werden kann). Die Sektion der drei Tiere bestätigte, dass die Epidermis neben der Rautenzeichnung schwach rot gefärbt war. Die Malpighischen Gefässe waren rosa, Darm und Fettkörper weiss, bzw. farblos.

66 CE: 11 Tiere. 4 blieben ohne Reaktion länger als 3 Tage am Leben. Die übrigen 7 reagierten deutlich durch Umfärbung. 4 von ihnen zeigten schon 1 Tag nach der Injektion im dorsalen Bereich neben der Rautenzeichnung die schmutzig olivgrüne Färbung, die bei normalen Tieren den Beginn der Umfärbung anzeigt (Färbungsstadium Ia). 2 Tage nach der Injektion hatten sie eine deutlich rötliche Färbung, die dem Färbungsstadium Ib normaler Tiere entsprach. Die anderen 3 Tiere erreichten das Färbungsstadium Ia am 2. Tage, eine von ihnen noch das Stadium Ib am 3. Tage. Sektionsergebnis: Bei allen 7 Tieren waren Epidermis und Malpighische Gefässe rot, bei einigen auch ein Teil des Darminhaltes in der Nähe der Einmündung der Malpighischen Gefässe. Der Fettkörper war in allen Fällen weiss.

330 CE: 3 Tiere. 2 davon überlebten die ersten 3 Tage nach der Injektion. Beide zeigten nach 3 Tagen eine Rotfärbung, die nicht in der Epidermis lokalisiert war und sich bis zur Sektion am 7. Tag nicht mehr verstärkte. Sektionsergebnis: Epidermis farblos. Fettkörper und Darminhalt rot.

1300 CE: 4 Tiere. Alle 4 änderten 2 Tage nach der Injektion ihre Farbe: Der vorher grüne Bereich wurde grau, nur im dorsalen Bereich schimmerte der Fettkörper rot durch. 2 der Tiere änderten auch ihre Form. Sie nahmen die Vorpuppenform an (Abb. 3). Die Vorpuppe ist hinten nicht spitz wie die Raupe sondern abgerundet. Die einzelnen Segmente sind stärker gegeneinander abgesetzt als bei der Raupe. (Abb.1d.)

3300 CE: 17 Tiere. 11 überlebten mehr als 3 Tage. Alle 11 zeigten dieselben Veränderungen von Farbe und Form, wie sie in der vorherigen Versuchsgruppe (1300) CE beschrieben wurden. Bei 6 dieser Tiere war der Übergang in die Vorpuppenform besonders ausgeprägt. Diese Tiere zeigten auch die für die Vorpuppe typischen Bewegungen. Diese bestehen nicht wie die der Raupe aus abwechselnder Streckung und Kontraktion der Segmente sondern wie bei der Puppe aus Rollbewegungen des Abdomens um seine Längsachse. Sektionsergebnis: Epidermis farblos. Darminhalt und Fettkörper rot.

6600 CE: 10 Tiere. 6 überlebten mehr als 3 Tage. Alle 6 färbten sich am 2. Tag schmutzig grau und nahmen die Vorpuppenform an. Nach 3 Tagen war äusserlich zu erkennen, dass sich die Cuticula von der Epidermis gelöst hatte. Zwischen beiden sammelte sich eine schwarze Flüssigkeit an. Sektionsergebnis: Bei 4 Tieren hatte sich der Darminhalt rot gefärbt, bei 2 davon auch der Fettkörper. Bei den anderen 4 Tieren war der Fettkörper weiss oder gelblich geblieben.

Die Ergebnisse mit reinem kristallisierten Hormon entsprachen den beschriebenen mit Hormonkonzentrat. Die Sterblichkeit der Versuchstiere war grösser:

13 CE: 4 Tiere, keines überlebte 3 Tage nach der Injektion.

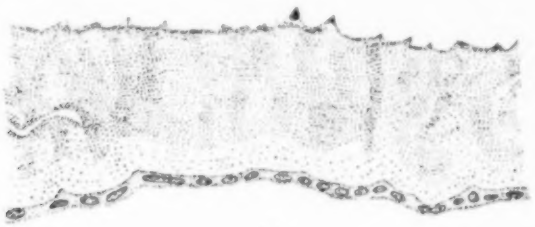
66 CE: 11 Tiere. 7 überlebten mehr als 3 Tage. Davon färbten sich 2 um. Eins erreichte das Stadium Ib, eins das Stadium Ia.

130 und 330 CE: Insgesamt 7 Tiere. 4 überlebten mehr als 3 Tage, 3 davon färbten sich um, erreichten aber nur das Stadium Ia.

VOL. 3 1959

Die histologischen Veränderungen nach der Injection von Ecdyson.

Begründung. Die Versuche mit Dosen von 3300 CE und 6600 CE hatten Reaktionen zur Folge die an das normale Vorpuppenstadium und die Puppenhäutung erinnern. Histologische Untersuchungen mussten zeigen, ob durch Injektion von Ecdyson tatsächlich histologische Veränderungen der Epidermis hervorgerufen werden, und wieweit diese den normalen Veränderungen vor der Puppenhäutung vergleichbar sind. Der normale histologische Zustand der



(a)

(b)

(c)

ABBILDUNG 6. Querschnitte durch die Epidermis. (a) Normales Tier, Stadium O: Plattenepithel mit fest anliegender Cuticula. (b) Normales Tier, Stadium IV: Stelzenepithel, Cuticula abgelöst, erste Schichten der neuen Cuticula an der Aussenseite der Epidermis. (c) Testabdomen nach Injektion von 6600 CE: Stelzenepithel, an der Aussenseite die ersten Schichten der neuen Cuticula, alte Cuticula abgelöst, zwischen beiden Exuvialflüssigkeit. Etwa 150:1.

Epidermis in den verschiedenen Färbungsstadien war bereits beschrieben worden (BÜCKMANN, 1953):

In der grünen fressenden Raupe, Färbungsstadium O, ist die Epidermis ein flaches Plattenepithel, dem die dicke Cuticula fest aufliegt (Abb. 6a). Dieser Zustand bleibt über die Umfärbung hinweg erhalten bis zum Beginn des Stadiums IV. Dann beginnt die Häutung im weiteren Sinne: Die Epidermiszellen durchlaufen eine Mitosenperiode. Dann strecken sich die Zellen, sodass nacheinander ein kubisches Epithel, und ein Zylinderepithel entsteht. Schliesslich strecken sich die Zellen so stark, dass sie seitlich den Kontakt miteinander verlieren und zu langen dünnen Protoplasmasträngen werden, es entsteht ein Stelzenepithel (Abb. 6b). Schon während sie sich strecken, bilden die Zellen an ihrer Aussenseite die ersten Schichten der Puppencuticula. Die Raupencuticula löst sich ab. Zwischen der alten und der neuen Cuticula sammelt sich die Exuvialflüssigkeit.

Methodik. Von den 10 zur histologischen Untersuchung nach BOUIN fixierten Testabdomina wurden jeweils die beiden vordersten Segmente, also das dritte und das vierte Abdominalsegment histologisch untersucht. Einbettung über Paraffin, Schnittrichtung quer zur Längsachse; Schnittdicke: 9 μ. Gefärbt wurde teils mit Haematoxylin DELAFIELD,

teils mit Haemalaun-Eosin. Einige Schnitte erhielten eine selektive Kernfärbung mittels der FEULGENschen Nuclealreaktion, die jedoch nach BOUIN-Fixierung nicht besonders gut wird.

Ergebnisse. Nach der Injektion von Ringerlösung blieb die Epidermis im gleichen histologischen Zustand wie in der grünen fressenden Raupe: ein flaches Plattenepithel mit fest aufliegender Cuticula. Dasselbe war der Fall nach allen Hormoninjektionen von bis zu 330 CE, also bei Tieren, deren Epidermis oder Fettkörper sich umgefärbt hatte. Nach Injektion von 6600 CE zeigten sich deutlich charakteristische Veränderungen im histologischen Zustand der Epidermis (Abb. 6c). Die Epidermis hat sich in ein typisches Stelzenepithel umgewandelt. An ihrer Aussenseite zeigen sich die ersten Schichten einer neugebildeten Cuticula. Die alte Cuticula hat sich abgelöst. Zwischen beiden befindet sich Flüssigkeit. Das histologische Bild entspricht dem eines normalen Tieres im Stadium IV. Nur bei einem der 5 histologisch untersuchten Tiere dieser Serie war die histologische Umwandlung der Epidermis nur bis zum Zylinderepithel fortgeschritten. Aber auch hier hatte sich die alte Epidermis abgelöst und die ersten Schichten einer neuen sind stellenweise deutlich zu erkennen. Der histologische Zustand entspricht dem eines normalen Tieres am Beginn des Stadiums IV.

Besprechung. Die Reaktionen auf die verschiedenen Hormonkonzentrationen entsprechen sehr genau verschiedenen Umfärbungsstadien, die in der normalen Entwicklung nacheinander eintreten. Die Reaktion auf die schwächsten Dosen 13 und 66 CE bestand in der Rötung der Epidermis im dorsalen Bereich neben der Rautenzeichnung. Gerade dort wird auch beim normalen Tier im Stadium Ia die Umfärbung zuerst erkennbar. Nach Injektion von 66 CE erreichte ein Teil der Tiere auch das Stadium Ib. Nach Injektion von 330 CE entfärbte sich die Epidermis; Fettkörper und Darminhalt wurden rot. Das entspricht dem normalen Färbungsstadium III, wenn sich Fettkörper und Darminhalt rot gefärbt haben, die Epidermis sich aber schon wieder entfärbt hat. Die Testabdomina erreichten dieses Stadium III, ohne dass sich vorher die Epidermis rot gefärbt hätte. Sie übersprangen die Stadien I und II auf diese Weise. Nach Injektionen von 3300 und 6600 CE reagierten die Testabdomina durch Übergang in die Vorpuppenform und Ablösung der alten Cuticula. Das entspricht dem normalen Stadium IV, in welchem die Puppenhäutung im weiteren Sinne in der Epidermis einsetzt. Die histologischen Befunde bestätigen, dass es sich bei der Ablösung der Cuticula nach Hormoninjektion nicht um rein pathologische Vorgänge handelt, sondern dass in der Epidermis die normalen typischen und sehr komplizierten Vorgänge der Puppenhäutung abgelaufen sind. Eindeutig pathologisch ist nur die schwarze Farbe der Exuvialflüssigkeit. Die abgelöste Raupencuticula scheint bei den Testabdomina auch nicht ganz so stark in sich selber aufgelöst zu sein, wie bei normalen Tieren im Stadium IV (vgl. Abb. 6b und 6c), was für eine etwas überstürzte Puppenhäutung spricht. Sonst aber haben diese Tiere durch das normale Färbungsstadium IV reagiert, ohne dass sich vorher die Epidermis und bei einem Teil auch der Fettkörper umgefärbt hätte. Die Tiere sind unter Überspringung der Färbungsstadien I–III in die Puppenhäutung übergegangen.

Die Reaktion auf die verschiedenen Hormondosen besteht also jeweils im Übergang in ein bestimmtes Stadium der normalen Entwicklung innerhalb von 3 Tagen und unter Überspringung normalerweise vorhergehender Stadien. Auf diesem betreffenden Zustand bleibt die Entwicklung nach 3 Tagen stehen. Je höher die Hormondosis ist, desto mehr Stadien werden übersprungen, desto später liegt also das erreichte Stadium in der normalen Entwicklung. Auf schwache Hormondosen erfolgt Umfärbung der Epidermis, auf stärkere Dosen Umfärbung des Fettkörpers und auf sehr starke Dosen Puppenhäutung.

Nachdem es damit gelungen ist, die Umfärbung von Epidermis und Fettkörper experimentell an Testabdomen durch Ecdyson auszulösen, kann kaum ein Zweifel mehr bestehen, dass auch im normalen Tier die Umfärbung durch dieses Hormon ausgelöst wird.

(6) *Vorzeitige Auslösung der Umfärbung bei jungen Larven*

Begründung. Die bisherigen Untersuchungen haben ergeben, dass die Umfärbung durch das Häutungshormon ausgelöst wird. Ausschüttungen des Häutungshormons erfolgen bei jeder Larvenhäutung. Es müsste also eigentlich auch schon in den jungen Larven bei jeder Larvenhäutung eine Umfärbung ausgelöst werden können. Tatsächlich färben sich aber niemals die jungen Larven bei den Larvenhäutungen um sondern nur die erwachsenen Larven vor der Puppenhäutung. Es war zu untersuchen, ob auch in jungen Larven die Umfärbung ausgelöst werden kann und warum das normalerweise bei den Larvenhäutungen nicht geschieht. Es war denkbar, dass die Hormonkonzentration bei den Larvenhäutungen nicht ausreicht für die Umfärbung. Das war aber unwahrscheinlich, weil sich ja gezeigt hatte, dass die Umfärbung zu ihrer Auslösung eine weit geringere Hormonmenge benötigt als die Puppenhäutung. Da die Umfärbung wahrscheinlich als ein erster Teilvorgang der Verpuppung aufzufassen ist (BÜCKMANN 1959), ist es viel wahrscheinlicher, dass sie, wie die gesamte Verpuppung, in der jungen Larve verhindert wird durch das Juvenilhormon der Corpora allata. In diesem Falle müssten sich junge Raupen nach Ausschaltung der Corpora allata umfärben können. Die Exstirpation der Corpora allata ist bei den empfindlichen Jungraupen von *Cerura vinula* bisher nicht gelungen. Es ist aber eventuell möglich, die Corpora allata einfach durch Dekapitierung auszuschalten, ohne damit zugleich die Wirkung des Gehirnhormons auf die Prothoraxdrüse und damit die Ausschüttung des Häutungshormons zu verhindern. Nach FUKUDA (1944) verpuppen sich nämlich junge Seidenraupen vorzeitig, anstatt sich zu weiteren Larvenstadien zu häuten, wenn sie in einem bestimmten Zeitraum dekapitiert werden. In diesem Zeitraum ist offensichtlich die Ausschüttung des Gehirnhormons schon erfolgt, während die des Juvenilhormons noch durch die Dekapitation verhindert werden kann.

Es sollte versucht werden, auf die gleiche Weise auch in jungen Larven von *Cerura vinula* die Corpora allata durch blosse Abschnürung des Kopfes auszuschalten. Dabei bestand die Hoffnung, die Umfärbung müsse sich auf diese Weise sogar noch leichter erzielen lassen als eine vorzeitige Puppenhäutung, da

sie schon durch eine geringere Menge des Häutungshormons ausgelöst wird. Sie könnte auch in solchen Fällen noch auftreten, wo die Ausschüttung des Häutungshormons durch die Dekapitation beeinträchtigt wurde. Um den geeigneten Schnürungszeitpunkt zur selektiven Ausschaltung der Corpora allata zu finden, mussten möglichst viele Larven in verschiedenen Zeitpunkten eines Häutungsintervalls geschnürt werden. Da die Schnürung zugleich eine vorzeitige Beendigung der Fressperiode zwischen den beiden Häutungen bedeutet, war in Kontrollserien zu prüfen, welche Reaktion schon allein durch diesen vorzeitigen Nahrungsentzug bewirkt wird.

Methodik. Die Untersuchungen wurden mit Larven des vierten (d.h. des vorletzten) Larvenstadiums durchgeführt. Die Dauer dieses Stadiums unter den Versuchsbedingungen (25°C) wurde bei einer Kontrollserie von 147 Tieren ermittelt. Weitere 69 Tiere wurden in 4 Gruppen ½, 1, 1½ und 2 Tage nach der dritten Larvenhäutung vom Futter genommen. Durch Abschnürung des Kopfes dekapitiert wurden insgesamt 97 Tiere, und zwar in folgenden Zeitabständen nach der dritten Häutung: ½ Tag (7 Tiere), 1 Tag (8 Tiere), 1½ Tage (45 Tiere), 2 Tage (31 Tiere) und 2½ Tage (6 Tiere). Die Tiere der Hunger- und Schnürungsexperimente wurden auf Häutungen, Umfärbungen und die Abgabe roten Exkretpigmentes hin kontrolliert und möglichst sofort nach Aussetzen des Herzschlages seziert zur Feststellung des Färbungszustandes der inneren Organe.

Ergebnisse. Die Dauer des vierten Larvenstadiums von der dritten bis zur vierten Larvenhäutung schwankte bei 147 Tieren zwischen 3 und 4½ Tagen. Sie betrug in Mittel $M = 3{,}7 \pm 0{,}02$ Tage (Standartabweichung $s = 0{,}3$ Tage). Schon 1½ Tage vor Eintritt der vierten Häutung, also im Mittel 2,2 Tage nach der dritten Häutung hören die Larven auf zu fressen und spinnen sich fest. Kurz darauf ist der Beginn der Häutungsvorgänge zu erkennen an der vorgeschobenen Kopfkapsel, aus welcher der Kopf zurückgezogen wird (vgl. v. BUDDENBROCK 1931).

Die *Hungerversuche* zeigen, dass nicht die gesamte normale Fressperiode von 2,2 Tagen zwischen den Häutungen erforderlich ist: Alle früher als 1½ Tage nach der dritten Häutung vom Futter genommenen Tiere starben ohne weitere Häutung, aber die nach 1½ Tagen vom Futter genommenen vermochten sich noch zu 77 Prozent zum vierten Male zu häuten, die nach 2 Tagen vom Futter genommenen zu 100 Prozent. Die Häutungen führten stets zu normalen Tieren des fünften Stadiums, das sich von dem vierten morphologisch stark unterscheidet (BÜCKMANN, 1953). Eine Umfärbung der Epidermis fand in keinem Falle statt. Dagegen gaben 9 Tiere rotes Exkretpigment aus dem Darm ab. Sie verteilten sich über alle 4 Versuchsgruppen, d.h. auch Tiere, die schon ½ und 1 Tag nach der dritten Häutung vom Futter genommen worden waren, vermochten Exkretpigment abzugeben. Die Sektion zeigte, dass sogar die meisten Tiere des Hungerversuches Exkretpigment gebildet hatten: Bei 58 der 69 Tiere war der Inhalt der Malpighischen Gefässe, sonst ein weisser Brei, rosa gefärbt. Bei 16 von ihnen war ausserdem der Darminhalt leuchtend rot oder orange gefärbt.

Auch die *Schnürungsexperimente* führten zur Bildung roter Exkretpigmente bei fast allen Versuchstieren. 28 Tiere aus allen Gruppen gaben rotes Pigment durch

den After nach aussen ab. 74 wurden seziert, 61 davon enthielten rotes Exkretpigment: Bei 7 Tieren war nur der Inhalt der Malpighischen Gefässe rosa gefärbt, bei insgesamt 54 war der Darminhalt leuchtend rot oder orange, bei 16 Tieren war beides der Fall.

Alle ½ und 1 Tag nach der dritten Häutung geschnürten Tiere starben ohne weitere Häutung. Von den 1½ Tage nach der dritten Häutung geschnürten häuteten sich 62 Prozent und von den noch später geschnürten 90 Prozent. Die Häutungen führten stets zu normalen Tieren des fünften Stadiums, abgesehen davon, dass, wie auch bei den Hungerversuchen, ein Teil der Tiere rotes Exkret abgab, was sonst bei keiner Larvenhäutung geschieht. *Drei der dekapitierten Tiere färbten sich um.* Es handelt sich um zwei nach 1½ Tagen und ein nach 2 Tagen geschnürtes Tier. Die Rotfärbung war deutlich ausgeprägt wie die normalerweise vor der Puppenhäutung erfolgende Umfärbung der erwachsenen Raupen (s. Farbphoto Bild 4). Alle drei Tiere starben, ohne sich noch zu häuten. Bei einem der drei Tiere zeigte die Sektion, dass sich nicht nur rotes Exkretpigment gebildet hatte, auch die Ganglien des Bauchmarks waren leuchtend rot gefärbt. Die übrigen Teile des Nervensystems waren dagegen normal farblos.

Besprechung. Ein überraschendes Ergebnis war die leichte Auslösbarkeit der Exkretpigmentbildung durch blossen Nahrungsentzug in jedem beliebigen Zeitpunkt des vierten Larvenstadiums. Normalerweise wird nur vor der Verpuppung und nach dem Schlüpfen des Falters rotes Exkret abgegeben. Möglicherweise bewirkt der Nahrungsmangel Veränderungen in Stoffwechsel, welche denen ähneln, die vor der Verpuppung zur Exkretpigmentbildung führen. Vielleicht beeinträchtigt der Nahrungsentzug auch die Abgabe des Juvenilhormons durch die Corpora allata. Dann wäre die Exkretpigmentbildung die empfindlichste Reaktion auf die Verschiebung des hormonalen Gleichgewichtes zuungunsten des Juvenilhormons, wie sie auch die Verpuppung und die weitere Metamorphose herbeiführt. Diese Fragen können erst durch weitere Untersuchungen geklärt werden. Auch die Rotfärbung der Ganglien bei einem der geschnürten Tiere kann vorläufig nicht gedeutet werden. Bei hunderten im Laufe mehrjähriger Untersuchungen sezierter Raupen wurde bisher niemals etwas derartiges beobachtet. Es ist auch unbekannt, ob die Rotfärbung eine Folge der Schnürung oder schon vorher vorhanden war.

Im Übrigen ergibt sich folgendes Bild über die Vorgänge im vierten Larvenstadium: Von den 3,7 Tagen, die es durchschnittlich dauert, sind die ersten 1½ die obligatorische Fressperiode. Nach ihrem Ablauf sind die Larven unabhängig von weiterer Nahrungsaufnahme zur vierten Häutung in der Lage. Die Dekapitation verhinderte 1 Tag nach der dritten Häutung 100 Prozent, 1½ Tage nach der dritten Häutung nur noch 38 Prozent und noch später 10 Prozent der Tiere an der vierten Häutung. Die Ausschüttung des Gehirnhormons, welche die vierten Häutung einleitet, muss also bei über 60 Prozent der Tiere 1½ Tage nach der dritten Häutung schon erfolgt sein. Der günstige Zeitraum für eine etwaige Ausschaltung der Corpora allata ohne Beeinträchtigung der Wirkung des Gehirnhormons muss direkt nach der Ausschüttung des letzteren liegen. Er war also im

VOL. 3 1959

Zeitraum 1½ bis 2 Tage nach der dritten Häutung zu vermuten. In diesem Zeitraum wurde überwiegende Teil der Versuchstiere dekapitiert, insgesamt 76 Tiere. Dennoch wurde in keinem Fall ein Schnürungszeitpunkt zwischen der Ausschüttung des Gehirnhormons und der des Juvenilhormons getroffen, denn in keinem Falle folgte eine vorzeitige Puppenhäutung. Das spricht dafür, dass im vierten Larvenstadium von *Cerura vinula* diese beiden Hormonausschüttungen einander in sehr kurzem Zeitabstand folgen, sich wahrscheinlich sogar zeitlich überschneiden. Die rote Umfärbung aber erfolgte in drei Fällen. Sie wird also tatsächlich durch Dekapitation bei jungen Larven ermöglicht. Es ist anzunehmen, dass in diesen Fällen die Dekapitation die Ausschüttung des Juvenilhormons rechtzeitig verhinderte, aber auch die des Gehirnhormons noch stark beeinträchtigte. So kam es zu einer unvollkommenen Aktivierung der Prothoraxdrüse und zur Ausschüttung einer nur geringen Menge Häutungshormon. Diese langte dann zur Auslösung der Umfärbung aber nicht mehr zu derjenigen der Häutung. Für die Möglichkeit, dass nach Abschnürung des Kopfes eine anomal geringe Menge Häutungshormon ausgeschüttet wird, sprechen schon frühere Befunde von KÜHN und PIEPHO (1936) und BÜCKMANN (1953), wo es nach Dekapitation zu Teilverpuppungen, bzw. zu Teilumfärbungen kam. Dass die vorzeitige Umfärbung der jungen Raupen nach der Dekapitation speziell auf der Ausschaltung der Corpora allata beruht, ist zwar nicht streng bewiesen, doch scheint die vorgetragene Annahme die plausibelste Deutung der Befunde zu sein.

Auf jeden Fall ist gezeigt, dass auch in jungen Larven die Umfärbung auslösbar ist. Da sie durch Dekapitation ermöglicht wurde, ist zu schliessen, dass sie normalerweise durch ein im Kopf gelegenes Zentrum verhindert wird. Dass dieses Zentrum die Corpora allata sind, ist sehr wahrscheinlich. Die Tatsache, dass in den Versuchen die Umfärbung statt der vierten Larvenhäutung erfolgte, spricht datür, dass sie auch in diesen jungen Raupen durch das Häutungshormon ausgelöst wurde.

DISKUSSION

Die experimentellen Ergebnisse vermitteln folgendes Bild von der *Auslösung der Umfärbung* vor der Puppenhäutung: Am Anfang des letzten Larvenstadiums ist eine Periode ununterbrochener Nahrungsaufnahme von 4 Tagen obligatorisch. Wird den Larven schon vorher die Nahrung entzogen, so vermögen sie sich nicht mehr normal umzufärben und zu verpuppen. Nach dieser Periode sind die Larven zur Umfärbung in der Lage, und dieselbe folgt, unabhängig davon, ob die Larven noch weiter fressen und wachsen oder nicht, durchschnittlich 5,6 Tage nach der letzten Larvenhäutung. Die Umfärbung wird ausgelöst durch einen stofflichen Faktor, der 6 Stunden vorher von einem thorakalen Zentrum abgegeben worden ist. Dies thorakale Zentrum muss seinerseits vom Kopf her aktiviert werden.

Durch Enthirnung kann ebenso wie durch Abschnürung des ganzen Kopfes die Aktivierung des thorakalen Zentrums verhindert werden, und die Umfärbung bleibt aus. Das im Kopf gelegene Zentrum, welches den Anstoss zur Umfärbung gibt, ist also sehr wahrscheinlich das Gehirn. Doppelte Schnürungen zeigen,

dass es die Umfärbung nicht direkt auslöst sondern eben über ein thorakales Zentrum. Einfache Schnürung hinter dem Thorax verhindert die Ausbreitung des umfärbungsauslösenden Faktors von dem thorakalen Zentrum her, und nur das vor der Ligatur gelegene Körperstück färbt sich um. Das hintere bleibt grün. In solchen grüngebliebenen Hinterstücken durchschnürter Raupen, lässt sich aber die Umfärbung der Epidermis auslösen durch Injektion kleiner Mengen des Häutungshormons α-Ecdyson. Stärkere Dosen dieses Hormons bewirken die Umfärbung des Fettkörpers und sehr grosse Dosen bewirken die Puppenhäutung ohne vorherige Umfärbung. Ein und dasselbe Hormon zeigt also in verschiedenen Konzentrationen qualitativ verschiedene Wirkungen. Nach diesen Ergebnissen ist der umfärbungsauslösende Faktor das Häutungshormon α-Ecdyson in geringer Konzentration. Das umfärbungsauslösende Zentrum muss demnach der Bildungsort dieses Hormons sein, also die Prothoraxdrüse. Das stimmt gut überein mit den Ergebnissen der Schnürungsexperimente, denenzufolge das umfärbungsauslösende Zentrum im Thorax gelegen ist und vom Gehirn aktiviert werden muss. Beides trifft ja für die Prothoraxdrüse zu.

VOL. 3 1959

Im Injektionsexperiment bestand die Reaktion auf verschiedene Dosen α-Ecdyson immer im Erreichen eines Zustandes, der einem Stadium der Normalentwicklung entsprach. Die dem betreffenden Stadium normalerweise vorhergehenden Entwicklungsstadien wurden übersprungen. Je stärker die Hormondosis war, ein desto fortgeschritteneres Stadium der Entwicklung wurde erzielt. Danach kann man sich die Ursache der Reihenfolge dieser Stadien in der normalen Entwicklung folgendermassen vorstellen: Die Konzentration des α-Ecdysons im Körper steigt allmählich an, sodass sie nacheinander ausreicht, um die einzelnen Stadien auszulösen, zunächst die Umfärbung der Epidermis, dann die des Fettkörpers, darauf das Wiederverblassen der Epidermis und schliesslich die Puppenhäutung. Diese Vorstellung entspricht der in der Einleitung unter 3(a) aufgeführten Hypothese nach PIEPHO. Für ihre Richtigkeit spricht auch die Tatsache, dass der Übergang vom Färbungsstadium II in das Stadium III, das Wiederverblassen der Epidermis, ebenfalls im hinteren Körperstück durch Abschnürung verhindert werden konnte. Wenn die angeführte Hypothese richtig ist, muss sich ja die Entwicklung praktisch auf jedem beliebigen Stadium durch Abschnürung weiterer Hormonzufuhr zum Stehen bringen lassen.

Über die *Bedeutung der Einwirkungsdauer* des Hormons für die Art der Reaktion, d.h. also über die Bedeutung des "Zeitfaktors der Hormonwirkung", auf den KARLSON, 1955, besonders hinwies, geben die vorliegenden Experimente nicht eindeutig Auskunft. Im Injektionsexperiment erfolgten die verschiedenen Reaktionen auf die verschiedenen Hormondosen immer innerhalb von 3 Tagen. Ausschlaggebend für die Art der Reaktion war also *nur die Hormonkonzentration, nicht die Einwirkungsdauer*. Dass die Entwicklung in jedem Fälle nach 3 Tagen stehenblieb, spricht für die Richtigkeit der Vermutung von KARLSON (1954), dass das Hormon im Körper bald inaktiviert wird. Im Experiment wurde immer die gesamte verwendete Hormondosis auf einmal injiziert. Dagegen müssen wir annehmen, dass im natürlichen Verlauf eine grosse Hormonkonzentration nicht

plötzlich wirksam wird, ohne dass schon vorher das Hormon in niedrigerer Konzentration wirkte. Mit der langsam ansteigenden Hormonkonzentration erhöht sich laufend seine Gesamteinwirkungsdauer. Auch wenn die Hormonkonzentration nicht die ganze Einwirkungsdauer über gleich hoch ist, kommt also doch der Zeitfaktor der Hormonwirkung in der normalen Entwicklung zur Geltung. Auf seinem Fehlen bei der plötzlichen Injektion hoher Hormondosen beruhen wahrscheinlich die pathologischen Begleiterscheinungen der experimentell ausgelösten Puppenhäutungen, vor allem die Schwärzung der Exuvialflüssigkeit und der relativ frühe Tod der Versuchstiere.

Eine weitere noch nicht geklärte Frage ist die nach der *Wirkung des Ecdysons auf die Atmungsfermente*. Vor der Puppenhäutung von *Cerura vinula* stellt die Ommochrombildung die früheste erfassbare Wirkung des Ecdysons dar. Bei der Imaginalhäutung von *Platysamia cecropia* fanden SCHNEIDERMAN und WILLIAMS (1953, 1954) als früheste erfassbare Reaktion eine Veränderung im System der Atmungsfermente. In der der Imaginalentwicklung vorausgehenden Puppendiapause ist die gesamte Atmung stark herabgesetzt und die Aktivität der einzelnen Atmungsfermente verändert. So fehlt vor allem die Cytochromoxydase fast vollständig. Wenn die Imaginalentwicklung einsetzt, werden als erstes diese Veränderungen rückgängig gemacht, und die Cytochromoxydaseaktivität steigt an. Es lag die Vermutung nahe, dass die erste und unmittelbare Wirkung des Häutungshormons diese Umstellung im System der Atmungsfermente sei. Dagegen ist nicht zu vermuten, dass dasselbe bei der Umfärbung von *Cerura* der Fall ist. Die Ommochrombildung kann wohl mit enzymatischen Veränderungen zussammenhängen, aber wohl kaum mit solchen Veränderungen der Aktivität der einzelnen Atmungsfermente wie sie der Imaginalhäutung von *Platysamia* vorausgehen. Auch bei *Platysamia* erfolgt sie nur vor der Imaginalhäutung, nicht vor der Puppenhäutung. Im Gegenteil: Im Augenblick der Puppenhäutung ist schon der umgekehrte Prozess im Gange, nämlich der starke Abfall der Cytochromoxydase—Aktivität, der zum Zustand der Puppendiapause überleitet. Soweit wir wissen, werden alle Häutungen durch das Häutungshormon, also Ecdyson ausgelöst. Der Anstieg der Cytochromoxydase-Aktivität ist aber nicht für alle Häutungen charakteristisch sondern nur für solche, die zugleich das Ende einer Diapause bedeuten. Es ist möglich, in ihm nicht die erste direkte Reaktion auf das Häutungshormon zu sehen, sondern eine indirekte, nämlich eine Folge der Ankurbelung des Stoffwechsels durch andere von dem Hormon eingeleitete Entwicklungsprozesse.

Interessant ist ein *Vergleich der verschiedenen Mengen von α-Ecdyson* die *zur Auslösung verschiedener Reaktionen* bei verschiedenen Arten benötigt wurden. Die empfindlichste bisher bekannte Reaktion ist die Puppariumbildung bei *Calliphora*. Sie ist zu erkennen an der Verhärtung und Bräunung der Larvencuticula. Wieweit auch die eigentliche Häutung ausgelöst wurde, ist nicht klar. Das isolierte *Calliphora*-Abdomen reagiert durch Puparisierung in 50–70 Prozent der Fälle auf eine *Calliphora*-Einheit (CE). Das ist die Definition der CE (KARLSON, 1955). Ein isoliertes *Calliphora*-Abdomen wiegt etwa 50 mg. Die Umfärbung der Epidermis von *Cerura vinula* konnte in 50 Prozent der Fälle ausgelöst werden

durch 66 CE. Das sind etwa 1,6 CE pro 50 mg Körpergewicht. Damit ist die Umfärbung von *Cerura vinula* neben der Puppariumbildung von *Calliphora* eine der empfindlichsten Reaktionen auf α-Ecdyson. Vielleicht hängt die grosse Empfindlichkeit dieser beiden Reaktionen damit zusammen, dass es sich nicht um eigentliche Häutungen handelt, sondern um die ersten eine Häutung vorbereitenden Prozesse. Die empfindlichste Reaktion durch eine vollständige Häutung fanden LÜSCHER und KARLSON (1958) bei der Termite *Kalotermes flavicolis*. Nach Injektion von 2,5 CE pro Tier häuteten sich über 50 Prozent der Versuchstiere. Bei der Wanze *Rhodnius prolixus* benötigte WIGGLESWORTH (1955) 100 CE pro Tier zur Auslösung einer Häutung. An Lepidopteren erprobte WILLIAMS (1954) das α-Ecdyson. Er konnte bei *Samia walkeri* die Imaginalhäutung isolierter Puppenabdomina auslösen durch 1667 CE pro Tier. Die vermutlich etwa gleich grossen isolierten Raupenabdomina von *Cerura vinula* benötigten zur Auslösung der Puppenhäutung 3300 CE. Das liegt in der gleichen Grössenordnung. Es ist aber doch die höchste bisher bei einem Insekt zur Auslösung einer Häutung gebrauchte Hormonmenge. Das kann natürlich mit physiologischen Artunterschieden zusammenhängen. Wahrscheinlich hängt es aber mit dem Zeitpunkt der Isolierung des Abdomens zusammen. Die verwendeten Larven von *Cerura vinula* waren ja noch vor der Umfärbung geschnürt worden. Diese liegt aber mehr als 10 Tage vor der Puppenhäutung. Wahrscheinlich würden sehr viel geringere Hormonmengen erforderlich sein, um Abdomina zur Puppenhäutung zu veranlassen, die erst kurz vor dem Übergang in die Vorpuppe isoliert worden sind. Es ist sogar bemerkenswert, dass die Puppenhäutung überhaupt bei so früh isolierten Abdomina ausgelöst werden konnte. Die Puppenhäutung anderer Lepidopteren, nämlich von *Galleria mellonella* und *Ephestia kühniella* konnte durch Ecdyson-Injektion überhaupt nur bei solchen Tieren ausgelöst werden, die erst im Vorpuppenstadium durchschnürt worden waren (HANSER und KARLSON, 1957). Nach einer Annahme von BECKER, 1941, fehlt in solchen Fällen vorher eine besondere Aktivierung der Epidermis, die diese erst zur Reaktion auf das Häutungshormon befähigt. Nach den neuen Ergebnissen an *Cerura vinula* ist es wahrscheinlich, dass diese Aktivierung nicht durch ein eigenes von BECKER angenommenes "Aktivierungshormon" erfolgt, sondern durch längere Einwirkung einer niedrigen Konzentration des Häutungshormons selber.

Dass nicht nur das Häutungshormon sondern auch das *Juvenilhormon die Umfärbung beeinflusst*, wird sehr wahrscheinlich durch die in Abschnitt 6 geschilderten Experimente. Sie zeigen, dass durch Dekapitierung die Umfärbung auch in jungen Larven herbeigeführt werden kann. Das spricht dafür, dass die Umfärbung, genauso wie die Puppenhäutung und alle weiteren Metamorphoseprozesse, in der jungen Larve verhindert wird durch das Juvenilhormon der Corpora allata. Das würde bedeuten, dass die Umfärbung durch dasselbe hormonale System und auf die gleiche Weise gesteuert wird wie die gesamte Metamorphose. Ähnlich wie es in Bezug auf die Auslösung durch das Häutungshormon der Fall ist, könnte sich die Umfärbung von der Puppenhäutung unterscheiden in der Menge des Juvenilhormons, die zu ihrer Hemmung erforderlich ist.

VOL. 3 1959

Die wichtigsten Folgerungen, über die *Physiologie der Verpuppung*, die sich aus den geschilderten experimentellen Ergebnissen ziehen lassen, sind folgende:

(1) *Der Zeitpunkt der Ausschüttung des Häutungshormons*

Wenn die Umfärbung durch eine niedere Konzentration desselben Hormons ausgelöst wird, das die Puppenhäutung bewirkt, muss dieses Hormon schon lange vor der kritischen Periode der Puppenhäutung im Körper vorhanden und wirksam sein, wenn auch eben in geringer Konzentration. Damit ergibt sich die Möglichkeit, dass nicht nur die Umfärbung sondern auch andere Vorgänge, welche schon der kritischen Periode der Puppenhäutung ablaufen, durch dieses Hormon ausgelöst werden. Das gilt vor allem für die Änderungen im Verhalten der Tiere: das Verlassen des Futters und den Kokonbau. Sie fallen zeitlich stets mit der Umfärbung zusammen, was besonders für eine Auslösung durch dasselbe Aktionssystem spricht. Auch konnte für den Kokonbau bereits gezeigt werden, dass er in jungen Larven durch das Juvenilhormon der Corpora allata unterdrückt wird (PIEPHO, 1950). Die Möglichkeit einer Auslösung durch niedrige Konzentration des Häutungshormons gilt ferner für die wachsende Determination der Epidermis zur Puppenhäutung, für die PIEPHO sie schon 1942 vermutete. Sie gilt schliesslich auch für alle anderen noch nicht analysierten physiologischen Vorbereitungen der Verpuppung, wie die Stoffwechselumsteuerung, die zur Bildung des roten Exkretpigmentes führt.

(2) *Die Erfolgsorgane des Häutungshormons*

Das sicher bekannte Erfolgsorgan für das Häutungshormon Ecdyson ist die Epidermis, ihre Reaktion besteht in den Häutungen. Die Verpuppung und die weitere Metamorphose bestehen aber in Veränderungen auch in allen inneren Organen. Es war zu vermuten, dass auch diese Veränderungen durch das Ecdyson bewirkt werden, wie schon der für dies Hormon verwendete Ausdruck "Metamorphosehormon" besagt (BUTENANDT und KARLSON, 1954). Die Versuche, in denen es gelang, in isolierten Abdomina von *Cerura* durch Ecdyson die Umfärbung des Fettkörpers auszulösen, zeigen nun direkt und besonders klar, dass das Ecdyson tatsächlich auch auf die inneren Organe wirkt, und dort Vorgänge steuert, die unmittelbar mit den epidermalen Häutungsprozessen nichts zu tun haben.

(3) *Die Wirkungsweise des Häutungshormons*

Die epidermalen Häutungsvorgänge, die durch Ecdyson ausgelöst werden, sind ausserordentlich komplex. Sie lassen sich nicht soweit analysieren, dass man sie auf einfache biochemische Reaktionen zurückführen könnte. Der einzige chemisch erfasste Teilvorgang, die Sklerotisierung der Cuticula, erwies sich als nicht direkt vom Ecdyson abhängig (KARLSON und SCHMID, 1955). Die Umfärbung dagegen besteht in der Bildung von Ommochromfarbstoffen. Von diesen ist der Weg ihrer Enstehung im Insektenkörper aus Tryptophan über verschiedene Zwischenstufen, und z.T. auch schon die chemische Struktur bekannt (BUTENANDT

und MITARBEITER, 1954, 1955). Das Auftreten der verschiedenen Ommochromfarbstoffe und die Veränderungen ihrer Mengen in den verschiedenen Färbungsstadien von *Cerura* konnten bereits quantitativ erfasst werden (BÜCKMANN, BIEKERT, NEUBERT und LINZEN, unveröffentlicht). Die Umfärbung stellt eine Wirkung des Ecdysons auf einen in seinen einzelnen Schritten gut erfassbaren chemischen Vorgang dar. Es besteht die Hoffnung, diese Wirkung noch genauer analysieren zu können, durch Ermittlung des direkten Angriffspunktes des Hormons in der vom Tryptophan zu den Ommochromen führenden Kette von Reaktionen.

Da angenommen werden muss, dass die Umfärbung auf Stoffwechselumsteuerungen beruht, die zur physiologischen Vorbereitung der Metamorphose gehören, gestattet sie gleichzeitig erste Rückschlüsse auf den Zeitpunkt und die Natur derartiger Prozesse und damit den allerersten Beginn aller Metamorphosevorgänge.

Acknowledgements—Herrn Dozenten DR. P. KARLSON, München, danke ich für die Überlassung von reinem Kristallisat und hochgereinigtem Konzentrat des Häutungshormons α-Ecdyson, durch die ein wesentlicher Teil der Arbeit erst ermöglicht wurde. Die Ergebnisse wurden belegt durch Farbaufnahmen mit Hilfe von Naheinstellgeräten, die die Deutsche Forschungsgemeinschaft Herrn Professor Dr. SCHALLER zur Verfügung gestellt hatte. Dafür und für eine Druckkostenbeihilfe, die den Abdruck von Farbphotos ermöglichte, danke ich der Deutschen Forschungsgemeinschaft.

VOL. 3 1959

LITERATUR

BECKER E. (1941) Über Versuche zur Anreicherung und physiologischen Charakterisierung des Wirkstoffs der Puparisierung. *Biol. Zbl.* **61**, 360–388.

BECKER E. (1942) Über Eigenschaften, Verbreitung und die genetisch entwicklungsphysiologische Bedeutung der Pigmente der Ommatin und Ommingruppe (Ommochrome) bei den Arthropoden. *Z. indukt. Abstamm.-u. Vererb.Lehre* **80**, 157–204.

BECKER E. und PLAGGE E. (1939) Über das die Puppariumbildung auslösende Hormon der Fliegen. *Biol. Zbl.* **59**, 326–351.

BERGOLD G. (1941) Eine Mikroinjektionsspritze und Mikrobürette bis zu 0.1 mm³. *Biol. Zbl.* **61**, 158–162.

BÜCKMANN D. (1952) Die Umfärbung von Schmetterlingsraupen vor der Verpuppung. *Naturwiss.* **39**, 213–214.

BÜCKMANN D. (1953) Über den Verlauf und die Auslösung von Verhaltensänderungen und Umfärbungen erwachsener Schmetterlingsraupen. *Biol. Zbl.* **72**, 276–311.

BÜCKMANN D. (1956) Die Umfärbung von *Cerura vinula* unter verschiedenen experimentellen Bedingungen. *Naturwiss.* **43**, 43.

BÜCKMANN D. (1956) Die Wirkung des Metamorphosehormons der Insekten. *Ber. üb. d. Hundertjahrfeier d. Deutschen Entomologischen Gesellschaft Berlin*, 238–249.

BÜCKMANN D. (1959) Farbwechsel als Teilvorgang der Metamorphose und seine Hemmung in jungen Schmetterlingsraupen. *Zool. Anz.* (Suppl.) **22**, 137–144.

BUDDENBROCK W. v. (1931) Untersuchungen über die Häutungshormone der Schmetterlingsraupen II. *Z. vergl. Physiol.* **14**, 415–428.

BUTENANDT A. und KARLSON P. (1954) Über die Isolierung eines Metamorphosehormons der Insekten in kristallisierter Form. *Z. Naturf.* **9b**, 389–391.

BUTENANDT A., SCHIEDT U., BIEKERT E., und KORNMANN P. (1954) Über Ommochrome, 1. Mitteilung: Isolierung von Xanthommatin, Rhodommatin und Ommatin C aus den Schlupfsekreten von *Vanessa urticae*. *Liebigs Ann.* **586**, 217–228.

BUTENANDT A., SCHIEDT U., und BIEKERT E. (1954) Über Ommochrome, 2. Mitteilung: Alkalischer und fermentativer Abbau von Xanthommatin und Rhodommatin. Alkalischer Abbau der Kynurenin-Seitenkette. *Liebigs Ann.* **586**, 229–239.

BUTENANDT A., SCHIEDT U., und BIEKERT E (1954) Über Ommochrome, 3. Mitteilung: Synthese des Xanthommatins. *Liebigs Ann.* **588**, 125–130.

BUTENANDT A., SCHIEDT U., BIEKERT E., und CROMARTIE J. (1954) Über Ommochrome, 4. Mitteilung: Konstitution des Xanthommatins. *Liebigs Ann.* **590**, 75–90.

BUTENANDT A. und NEUBERT G. (1955) Über Ommochrome 5. Mitteilung: Xanthommatin, ein Augenfarbstoff der Schmeissfliege *Calliphora erythrocephala*. *Hoppe-Seyl. Z.* **301**, 109–114.

BUTENANDT A. und BECKMANN R. (1955) Über Ommochrome, 6. Mitteilung: zur Genese von Rhodommatin und Ommatin C. *Hoppe-Seyl. Z.* **301**, 115–117.

CASPARI, E. und PLAGGE E. (1935) Versuche zur Physiologie der Verpuppung von Schmetterlingsraupen. *Naturwiss.* **33**, 751.

FUKUDA S. (1940) Hormonal control of moulting and pupation in the silkworm. *Proc. imp. Acad. Japan* **16**, 417–419.

FUKUDA S. (1944) The hormonal mechanism of larval moulting and metamorphosis in the silkworm. *J. Fac. Sci. Tokyo Univ. IV* **6**, 477–532.

HANSER G. und KARLSON P. (1957) Über die Wirkung des Metamorphosehormons auf die Epidermis von *Ephestia-Dauerraupen*. *Biol. Zbl.* **76**, 129–141.

KARLSON P. (1955) Biochemische Probleme der Insektenmetamorphose. *Zool. Anz.* Suppl. **18**, 68–85.

KARLSON P. (1956) Biochemical studies on insect hormones. *Vitam. and Horm.* **14**, 227–266.

KARLSON P. und BÜCKMANN D. (1956) Experimentelle Auslösung der Umfärbung bei *Cerura*—Raupen durch Prothorakaldrüsenhormon. *Naturwiss.* **43**, 44–45.

KARLSON P. und HANSER G. (1953) Bildungsort und Erfolgsorgan des Puparisierungshormons der Fliegen. *Z. Naturf.* **8b**, 91–96.

KARLSON P. und SCHMID H. (1955) Über die Tyrosinase der *Calliphora*-Larven. *Hoppe-Seyl. Z.* **300**, 35–41.

KARLSON P. und WECKER E. (1955) Die Tyrosinaseaktivität während der Puppariumbildung von *Calliphora erythrocephala*. *Hoppe-Seyl. Z.* **300**, 42–48.

KÜHN A. und PIEPHO H. (1936) Über hormonale Wirkungen bei der Verpuppung der Schmetterlinge. *Nachr. Ges. Wiss. Göttingen* **2**, 141–154.

KÜHN A. und PIEPHO H. (1938) Die Reaktion der Hypodermis und der Versonschen Drüsen auf das Verpuppungshormon bei *Ephestia kühniella* Z. *Biol. Zbl.* **58**, 12–51.

LÜSCHER M. und KARLSON P. (1958) Experimentelle Auslösung von Häutungen bei der Termite *Kalotermes flavicolis* (Fabr.). *J. Ins. Physiol.* **1**, 341–345.

PIEPHO H. (1939) Über den Determinationszustand der Vorpuppenhypodermis bei der Wachsmotte *Galleria mellonella* L. *Biol. Zbl.* **59**, 314–326.

PIEPHO H. (1940) Über die Hemmung der Verpuppung durch Corpora allata, Untersuchungen an der Wachsmotte *Galleria mellonella* L. *Biol. Zbl.* **60**, 367–393.

PIEPHO H. (1942) Untersuchungen zur Entwicklungsphysiologie der Insektenmetamorphose. Über die Puppenhäutung der Wachsmotte *Galleria mellonella* L. *Arch. EntwMech. Org.* **141**, 500–583.

PIEPHO H. (1950) Über die Hemmung der Falterhäutung durch Corpora allata. Untersuchungen an der Wachsmotte *Galleria mellonella* L. *Biol. Zbl.* **69**, 261–271.

PIEPHO H. (1950) Hormonale Grundlage der Spinntätigkeit bei Schmetterlingsraupen. *Z. Tierpsychol.* **7**, 424–434.

PIEPHO H. (1951) Über die Lenkung der Insektenmetamorphose durch Hormone. *Zool. Anz.* Suppl. **15**, 62–76.

PLAGGE E. (1938) Weitere Untersuchungen über das Verpuppungshormon der Schmetterlinge. *Biol. Zbl.* **58**, 1–12.

SCHARRER B. (1952) Über neuroendokrine Vorgänge bei Insekten. *Pflüg. Arch. ges. Physiol.* **255**, 154–163.

SCHARRER B. (1955) Hormones in invertebrates in *The Hormones*, III, 57–95, New York 1955.

SCHNEIDERMAN H. A. und WILLIAMS C. M. (1953) The physiology of insect diapause VII. The respiratory metabolism of the *Cecropia* silkworm during diapause and development. *Biol. Bull. Woods Hole* **105**, 320–334.

SCHNEIDERMAN H. A. und WILLIAMS C. M. (1954) The physiology of insect diapause VIII. Qualitative changes in the metabolism of the *Cecropia* silkworm during diapause and development. *Biol. Bull. Woods Hole* **106**, 210–229.

SCHNEIDERMAN H. A. und WILLIAMS C. M. (1954) The physiology of insect diapause IX. The cytochrome oxidase system in relation to the diapause and development of the *Cecropia* silkworm. *Biol. Bull. Woods Hole*, **106**, 238–252.

WIGGLESWORTH V. B. (1934) The physiology of ecdysis in *Rhodnius prolixus* (Hemiptera). II. Factors controlling moulting and "metamorphosis". *Quart. J. micr. Sci.* **77**, 191–222.

WIGGLESWORTH V. B. (1954) *The Physiology of Insect Metamorphosis*, Cambridge, Univ. Press.

WILLIAMS C. M. (1951) Biochemical mechanisms in insect growth and metamorphosis. *Fed. Proc.* **10**, 546–552.

WILLIAMS C. M. (1952) Physiology of insect diapause IV. The brain and prothoracic glands as an endocrine system in the *Cecropia* silkworm. *Biol. Bull. Woods Hole* **103**, 120–138.

WILLIAMS C. M. (1954) Isolation and identification of the prothoracic gland hormone of insects. *Anat. Rec.* **120**, 743.

VOL. 3 1959

PAPERS TO BE PUBLISHED IN FUTURE ISSUES

K. VAN ASPEREN: Distribution and substrate specificity of esterases in the housefly (*Musca domestica* L.)

K. MARUYAMA: Insect actomyosin. A flow birefringence study

ZWI H. LEVINSON and ERNST D. BERGMANN: Vitamin deficiencies in the housefly produced by antivitamins

R. L. PATTON, JOANN GARDNER, and ANN D. ANDERSON: The excretory efficiency of the American cockroach

T. YAMASAKI and T. NARAHASHI: Electrical properties of the cockroach giant axon

P. W. MILES: The salivary excretions of a plant-sucking bug *Oncopeltus fasciatus* Dall. (Heteroptera: Lygaeidae)—I. The types of secretion and their roles during feeding

E. OHNISHI: Studies on the mechanism of tyrosinase activation in the housefly *Musca vicina* M.

TERUHIKO HOSOI: Identification of blood components which induce gorging of the mosquito

A. E. TREAT and K. D. ROEDER: A nervous element of unknown function in the tympanic organs of moths

G. HOYLE: The neuromuscular mechanism of an insect spiracular muscle

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 191 *to* 218. *Pergamon Press Ltd., London. Printed in Great Britain*

IDENTIFICATION OF BLOOD COMPONENTS WHICH INDUCE GORGING OF THE MOSQUITO*

TERUHIKO HOSOI

Biological Laboratory, The Tokyo Institute of Technology, Tokyo, Japan

(*Received* 14 *July* 1958)

Abstract—The peculiar stimulation of mosquitoes by host blood, which usually ensures gorging and subsequent dispatch of the food to the midgut, may be supposed to be either tactile or gustatory. Having examined various properties of dietary blood for their effects upon the feeding responses of *Culex pipiens* var. *pallens* Coquillett, evidence has been obtained that the blood stimulants are predominantly chemical, and that the responsible substance or substances must be some components of the erythrocytes as well as of other living cells, rather than the fluid part of the blood, but that it is neither haemoglobin nor any other compounds heretofore suspected. The blood stimulus, though exceedingly acceptable to the mosquito, differs qualitatively from that of sugars. NaCl and similar inorganic chlorides in adequate concentrations also exhibit a slight degree of the blood-like stimulation. They are obviously rejected at higher concentrations. Osmotic pressure has no fundamental influence upon sugar feeding, but it can affect blood feeding, since hypotonic solutions of haemolysed erythrocytes are refused by the insect if they are not sweetened with sugar. Guided by such findings, it was possible to extract from ox blood cells a substance possessing the action of the blood stimulus. Through ion-exchange chromatographic separation this was identified as adenylic acid. Comparison of the effects of several nucleotides and their related compounds suggests that adenosine-5′-phosphates of blood cells afford the main stimulus which promotes gorging of the mosquito on a blood meal. Acceptability of the other compounds is also discussed in relation to their chemical structure.

VOL. 3 1959

INTRODUCTION

In mosquitoes two types of food are commonly known, i.e. blood and sugary fluids, of which the former, when gorged under natural circumstances, is dispatched primarily to the midgut, whereas the latter goes to the crop. Despite the greater or lesser divergence which might appear in the above sequence under laboratory conditions, recent authors are generally in agreement with Bishop and Gilchrist (1946), who state that in *Aedes aegypti* it is the nature of the food and not the method of feeding which determines whether the food goes into the stomach or diverticula (Trembley, 1952; Day, 1954; Hosoi, 1954a).

This, however, does not solve the problem how mosquitoes discriminate between different foods and deal with it suitably. Thus in 1954 Day and the

* A short communication of this work is to appear in *Nature* under the heading "Adenosine-5′-phosphates as the stimulating agent of blood to induce gorging of the mosquito".

present writer came to somewhat divergent opinions about the 'switching mechanism' of food ingestion. Later investigations to be described elsewhere led the writer to agree with DAY in recognizing that some of the buccal sensilla receive either the sugar or blood stimulus or both, discharging impulses which eventually reach the cardiac sphincter via the nerve centre and control its relaxation. In the same work further evidence is given in support of the writer's view that the tip of the labium also detects sugars but not blood; whereas the labrum is no longer considered as bearing any specific chemoreceptors. These conclusions represent partial confirmation of the fact previously observed by FRINGS and HAMRUM (1950) that the labellum and tarsi are the loci of contact chemoreceptors in adult *Aedes aegypti*.* It is because of this complexity in the mosquito's feeding mechanisms that the earlier contention by MACGREGOR (1930, 1931) seems to turn out once more to be tenable in its revised form; i.e. in certain instances the method of feeding evidently does alter the destination of food. For instance, in the presence of a strong sugar stimulus on the labellum of *Culex pipiens* var. *pallens* Coquillett, sugar-free blood presented to the unsheathed fascicle passes, not to the midgut, which is the normal destination of the meal, but directly to the crop (HOSOI, unpublished).

Leaving aside such complications, it is evident that the dietary blood exerts two peculiar effects upon mosquitoes; first, it causes engorgement without any aid from additional sugars; secondly, it is readily drawn into the midgut. The facts indicate that the stimulants afforded by blood are qualitatively different from those afforded by sugars. Current opinion is inclined to attribute them to blood cells rather than to plasma. So far as the writer is aware, however, no convincing accounts have ever been given of the nature of the stimulating principle of blood.† FISK (1950) was unable to feed *Aedes aegypti* on artificial foods provided with one or more factors from the blood. BISHOP and GILCHRIST (1946) noted the significant effectiveness of their (!) haemoglobin. In contrast, DAY (1954) appeared to ascribe the effect to the particulate property of blood cells. HOSOI (1954a) was not conclusive about whether tactile or chemical stimuli elicited the responses.

Investigations were therefore undertaken in an attempt to determine which blood factors mosquitoes accept eagerly and yet discriminate clearly from sugars. The effectiveness of substances was tested with *Culex pipiens* var. *pallens* Coquillett by one or more methods of feeding, and the experiments were carried out concurrently with those designed particularly for analyses of the 'switching mechanism'. These analyses were concerned largely with loci and sensory acuity of the receptors of contact stimuli. For the sake of avoiding confusion, however, the present account will be confined to elucidation of the first problem.

* The writer is indebted to Professor H. FRINGS of the Pennsylvania State University, who kindly notified him about this article and reproduced a copy of it for his use.

† That none has, is also affirmed by Professor V. G. DETHIER in a personal communication dated 24 February 1958.

GENERAL METHODS

The mosquito. A laboratory maintained strain of *Culex pipiens* var. *pallens* Coquillett furnished the sole source of test animals. It was chosen because of its thoroughly studied biting behaviour in relation to the conditions of breeding (Hosoi, 1954b). Unless otherwise stated, adult females raised at 20–25°C were allowed to feed on a 2 per cent solution of glucose for indefinite periods ranging from 2 days to 4–5 weeks before they were sugar-starved for subsequent use. Usually they were not employed repeatedly, a precaution against the possibility of their response changing as a consequence of a feeding test.

Feeding techniques. Test liquids were offered to the mosquito from cotton-wool saturated with them. In earlier experiments a voluntary feeding technique was applied to caged animals. It was soon abandoned and replaced by a kind of forcible feeding which had the following advantages: (1) elimination of variance derived from tactic responses; (2) possibility of terminating the feeding, if desired, within a few minutes, thus minimizing evaporation or sedimentation of the food; and (3) possibility of direct observation upon the act of drinking and timely determination of the food destination. One disadvantage was the impossibility of employing a large number of the insects while the experimental conditions were kept nearly constant.

A mosquito to be fed forcibly was imprisoned singly in a pencil-shaped glass tube so as to allow protrusion of the proboscis through the narrower opening of the tube. The tube was stoppered at the rear end of the insect with a cotton-wool plug fastened to a wire rod, which prevented any accidental avoiding movements of the animal during the test. The tube was then mounted on a slide glass with the aid of a rubber band, placed under a dissection microscope, and the test liquid was brought into contact with the tip of the proboscis, being warmed previously to an initial temperature of 40–50°C. Although no effort was made to unsheathe the stylets artificially, as did MacGregor (1930), the above technique could nevertheless be regarded as ensuring forcible feeding, since the mosquito was obliged to taste the offered liquid. Participation of the labellar sense organs in reception of the food stimuli did not cause much difficulty in judging the result for the present purposes, since these organs are almost insensitive to the blood stimulus. Gorging, if it took place with full vigour, was accomplished within a minute or two. In colder seasons the entire apparatus was placed inside of a frame-work supporting a cellophane sheath, within which atmospheric temperatures of 25–30°C could be constantly maintained by electric heating.

Judgment of effectiveness. The stimulative power of test liquids was measured essentially by the method developed by von Frisch (1934), ratios of positive responses as well as grades of crop or midgut loads being taken as indices of acceptability. Under the present conditions, almost every mosquito drank a given liquid at least in a very small amount, irrespective of whatever factors it contained, and a trace of it almost invariably went to the midgut. For this reason, acceptance of a liquid was mainly determined by the ratio of the animals which fed to repletion. The grade of satiation was estimated by observation on abdominal distension. Although in engorged females the destination of the food was roughly discernible from outside, it was confirmed by dissection whenever this procedure seemed necessary. Occurrence of full midguts in a significant number was routinely regarded as an indication of the presence of blood-like stimulants in the test liquids. In some comparative studies, numbers of partially fed animals were also assessed to supplement the data.

Material. The investigations were carried out along two parallel lines. The main course was directed to analyses of blood components and subsequent isolation of an acceptable compound or compounds. The other was aimed at providing liquids capable of simulating the stimulatory effect of blood. Experiments of the first category were performed almost exclusively with citrated ox blood. Detailed descriptions for the preparation of the test liquids will be given in the following sections together with the results.

VOL. 3 1959

RESULTS AND DISCUSSION

A. *Feeding Responses to Various Contact Stimulants*

1. *Distilled water, salines, and glucose*

During the beginning of this work it was not certain that blood and sugars were the only substances acceptable to the mosquito. FÜLLEBORN (1908, 1932) asserted that under forced conditions *Anopheles maculipennis* imbibes, occasionally until satiation, solutions of a great many compounds, indiscriminately. Since blood contains a variety of inorganic salts in addition to a small amount of glucose, some of them might possibly be responsible for the stimulatory action. In view of this the following materials were examined either singly or in combinations by both the voluntary and forcible techniques of feeding: distilled water, 0·15 M NaCl, 0·15 M KCl, 0·1 M $CaCl_2$, Ringer, and 2–5 per cent glucose.

Altogether 389 females were thus fed, giving rise to 142 cases of engorgement (with a small number of partial feeding included). Apparently the forcible method gave slightly better results than the voluntary, especially with glucose-free solutions; but the differences were not highly significant. No mosquitoes fed to repletion on distilled water, whereas small percentages did so on some of the glucose-free salines. In such instances, a solution of KCl or NaCl seemed preferable to that of $CaCl_2$ or Ringer, though not much could be inferred from the differences on account of the probable heterogeneity in the nutritional conditions of the animals tested. In all, 20 out of 173 took relatively large amounts of the sugar-less solutions of KCl or NaCl, which distended the midgut at least partially, and the crop to varying degrees. A full midgut in conjunction with an almost empty crop was demonstrated in only three, or less than 2 per cent of the animals exposed; one of these gorged KCl and the other two NaCl. In marked contrast, all the liquids sweetened with glucose caused high proportions of full feeding, sometimes attaining 100 per cent, and the bulk of the food invariably went to the crop.

It seems likely that the acceptance of NaCl and similar chlorides is not dependent upon abnormal conditions of the mosquito but upon a definite response to these compounds themselves, and further evidence is given below (Section B, 2). There are two factors which possibly account for the relative ease of saline ingestion as compared with distilled water. Either osmotic pressure of the liquid food influences the act of aspiration (Section B, 3), or the salts are tasted by the mosquito, or both factors may be concerned. Although most of the electrolytes thus far tested on insects are rejected, some of them are known to become more acceptable than distilled water at subliminal concentrations (DETHIER and CHADWICK, 1948). BUTLER (1940) has confirmed this with the honeybee and FRINGS (1946) with the cockroach. Allowing, by analogy, that the above stimulation of the mosquito by salines is gustatory, the modality should be different from that of sugar, since the salt solutions readily enter the midgut. The result is in accord with the observation by KADLETZ and KUSMINA (1929), who also stated that NaCl leads a blood meal to the midgut. In stimulation of the

tarsal receptors of a butterfly by NaCl, MORITA *et al.* (1957) successfully recorded trains of sensory impulses distinct in their pattern from those elicited by sucrose. As regards the destination in the mosquito, the salines bear a resemblance to blood; nevertheless, their stimulative power seems hopelessly small in comparison with that of blood. In fact, they are seldom taken to repletion; and if gorging takes place at all, it is accomplished only with a considerable delay.

2. *Blood and its components*

In view of the fact that *Stomoxys* and *Lyperosia* readily gorge on blood serum (KRIJGSMAN, 1930; KRIJGSMAN and WINDRED, 1930), it would seem somewhat strange that mosquitoes feed reluctantly on plasma or serum (BISHOP and GILCHRIST, 1946; DAY, 1954; HOSOI, 1954a). Further examinations were, therefore, made on blood and its major components. The following meals were presented either by voluntary or forcible techniques of feeding: (1) citrated ox whole blood; (2) suspensions of thrice-washed ox blood cells in plasma, serum, Ringer, 0·15 M NaCl, or 0·15 M KCl; and (3) ox plasma or serum alone. In each series, aliquots were made up to 2 per cent solutions with respect to glucose. In all, 865 females were exposed and 627 fed to repletion. The trends of gorging activity are represented in Fig. 1(a) and (c).

VOL. 3 1959

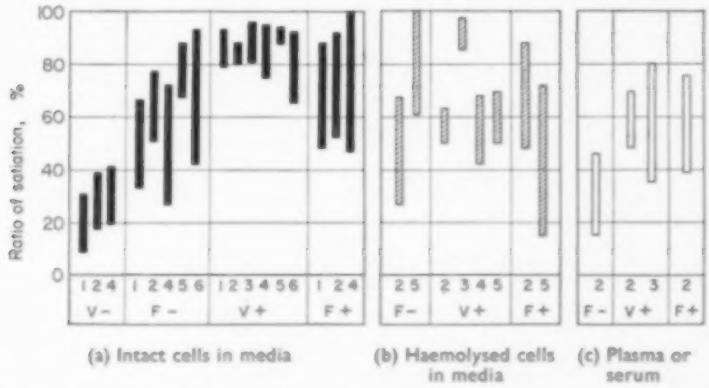


FIG. 1. Comparisons of the ratios of the satiate/exposed mosquitoes fed on various preparations of ox blood.

Confidence limits of the percentages taken at a 0·05 × 2 level of significance are illustrated. Intact (a) or haemolysed (b) blood cells mixed with media in equal parts, besides cell-free plasma or serum (c), are fed either voluntarily (*V*) or forcibly (*F*), and with (+) or without (−) addition of 2 per cent glucose. The media are as follows: (1) plasma (as whole blood), (2) plasma, (3) serum, (4) Ringer, (5) 0·15 M NaCl, and (6) 0·15 M KCl.

Evidently there exists a kind of resistance or difficulty for the insect to detect blood offered in the form of a free liquid, which is indicated by the significantly lower percentages of satiation for the voluntary feeding as compared with the forcible one (Fig. 1(a): *V*− vs. *F*−). A similar difference is also experienced with cell-free plasma although it is not shown in Fig. 1. That failure

to eat is not due to the lack of attractive odours in blood or serum, but due to the lack of some preliminary contact stimulation, is manifest from the comparison of the voluntary feedings in the presence or absence of glucose (Fig. 1(a): $V-$ vs. $V+$). Addition of 2 per cent glucose greatly improves the feeding activity on any liquids regardless of the method of presentation, thus eliminating the initial difficulty of aspiration. Whether the facilitation arises from the tarsi or the labellum was not determined, though both seem probable. The forcible feeding may also exert a trigger action upon the labellum in place of glucose.

Under the forced condition, the mosquito imbibed glucose-free suspensions of blood cells nearly as readily as sweetened ones (Fig. 1(a): $F-$ vs. $F+$). In contrast, sugarless plasma was not gorged so avidly as sweetened plasma under similar conditions of feeding (Fig. 1(c): $F-$ vs. $F+$). Moreover, the bulk of the ingested plasma seldom went to the midgut even in the absence of glucose, and never when it contained sugar, regardless of the method of feeding; whereas the cell suspensions consistently filled up the midgut in the absence of sugar, and seldom failed to do so even in the presence of 2 per cent glucose, again regardless of the method of feeding. Except for the presence or absence of sugar, usually no significant differences depending upon the nature of the suspending media appeared. It is true that the tendency of crop-filling was increased by the presence of glucose; nevertheless, the degree of this increase was insignificantly small in the case of the cell suspensions. For instance, only 7 out of the 469 satiated on sweetened blood cells exhibited fully distended crops accompanied by almost empty midguts. All of the 98 animals satiated on glucose-free cells had fully distended midguts. Conversely, in all of the 53 animals satiated on sweetened plasma or serum, the largest proportion of the liquid entered the crop.

It is manifest, therefore, that the stimulatory effect characteristic of blood is mainly dependent upon the properties of the cells and least upon plasma. In this respect, mosquitoes are evidently dissimilar to the previously mentioned haematophagous flies. On the other hand, a number of discordant results have been reported on the destination of blood in mosquitoes, especially when the blood is mixed with honey or sugars. Besides the differences in inclination of divergent species of insects, as particularly studied by ROY and GHOSH (1940) and TREMBLEY (1952), discrepancies would depend as much on the relative intensities of various stimuli as on the way the mosquitoes are fed. The problem will be considered again later (Sections B, 2 and 4).

3. *Fractions of erythrocytes*

Haeme and oxygen-carrying capacity of erythrocytes. Several lots of mosquitoes were subjected to mixtures of either intact or laked ox erythrocytes and plasma or Ringer in all the possible combinations, with haemoglobin converted to either carboxy- or met-haemoglobin in each aliquot. The results of feeding with these materials showed no divergence from the usual consequence of blood feeding, except that with carboxyhaemoglobin digestion of the blood was arrested until this became freed of carbon monoxide. Free haemin also exhibited no sign of

particular stimulation when it was presented to the animals in a 0·1 per cent solution in ox blood plasma.

Effect of haemolysis. Tests on haemolysed ox erythrocytes were performed simultaneously with those on the intact cells described previously (Section A, 2). Haemolysis was accomplished in three ways: (1) addition and subsequent removal of ether, (2) mixing the cells with distilled water followed by addition of NaCl or other suitable salts to adjust the osmotic pressure to a normal level, and (3) rapid freezing and thawing. Some 500 females were fed on mixtures of such laked cells and various media, mostly by the voluntary technique and in the presence of 2 per cent glucose.

The greater part of the results is summarized in Fig. 1(b). Altogether 233 out of the 372 exposed mosquitoes were satiated, most of them having a full midgut and none a distended crop only. In a word, the stimulative power of erythrocytes was on the average not affected significantly by haemolysis. However, discordant results were not infrequently encountered, and the percentage satiated sometimes fell remarkably. In some instances the reduction was probably derived from unsuitable preparation of the test liquids, say, contamination by a trace of ether or inadequate adjustment of osmotic pressure, and in the others from the physiological condition of the mosquitoes. All of these being eliminated, there still occurred certain degrees of fluctuation in the percentage. Since the laked blood showed a tendency to reduce its stimulative power during a period of prolonged standing, one cause of the instability might have been concerned with a kind of ageing phenomenon. Under what conditions this was likely to appear remained obscure.

VOL. 3 1959

Stroma, endosoma, haemoglobin, and globin. BISHOP and GILCHRIST (1946) have fed *Aedes aegypti* on 'haemolysed blood' or 'haemoglobin' of chick. Judging from their descriptions, these words imply one and the same thing, i.e. the stroma-free content of the erythrocytes, which the present writer will call 'endosoma'. According to the above authors, 'haemoglobin' is ingested to a lesser degree than the intact erythrocytes when fed through membranes, but definitely passes to the midgut; it is very rarely ingested from open drops, but passes mainly to the midgut. It therefore seems that the endosoma retains a considerable degree of the stimulus afforded by intact erythrocytes; consequently the stimulus must be more chemical than mechanical. An alternative, proposed by DAY (1954), is that the particulate nature of the blood cells is more important than the chemical one; in fact, in DAY'S experiments haemoglobin was dispatched to the crop.

During the study on egg production of the mosquito, the writer had opportunities of feeding the insect on components of ox erythrocytes such as their ghosts or stroma, endosoma, haemoglobin, and globin. Some of the engorged animals were dissected immediately after the meal to determine the distribution of ingested food. The ox stroma was prepared by the method of LETSCHE (1924) and served in a mixture with an equal part of plasma or 0·15 M NaCl. The ox haemoglobin was obtained in the form of crystalline oxyhaemoglobin by the

method of KIKUCHI (1953). The procedure also permitted one to provide a stroma-free solution of erythrocyte endosoma, which was subsequently dried under a reduced pressure. The ox globin was prepared first by the method of ANSON and MIRSKY (1930) and later according to KISTLER *et al.* (1953). Both the haemoglobin and globin were found in part denatured. All the latter three components were made up to 5–20 per cent solutions in plasma or distilled water. An equal part of a stroma suspension was also mixed with an aliquot of each solution thus obtained. At all times the test liquids contained 2 per cent glucose to allow for voluntary feeding of the mosquito. It was impossible to ensure that the liquids were osmotically isotonic with normal ox blood, although the desirability of this was realized.

Altogether 1382 mosquitoes were exposed to the liquids, yielding 917 cases of satiation, out of which 389 were dissected to demonstrate 99 fully distended midguts. As would naturally ensue from the feeding technique, any test caused a fairly high percentage satiation (Fig. 2(a)). Inconsistencies appeared among the ratios of midgut filling not only for different components of erythrocytes but also

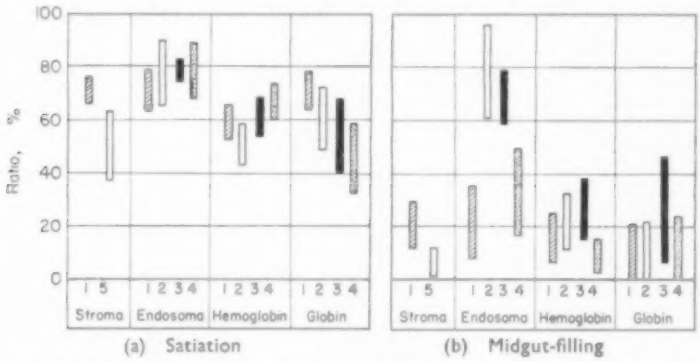


FIG. 2. Relative effects of erythrocyte components measured by the ratios of the satiate/exposed mosquitoes (a), and by those of the midgut-filled/satiate mosquitoes dissected (b). Confidence limits of the percentages taken at a 0·05 × 2 level of significance are illustrated. The animals are fed voluntarily on the designated components mixed with the following media containing 2 per cent glucose as well: (1) plasma, (2) distilled water, (3) plasma plus stroma, (4) distilled water plus stroma, and (5) 0·15 M NaCl.

for different preparations of the same component (Fig. 2(b)). On the whole, the ratios are greatest for endosoma and apparently least for globin. In some instances the endosoma is comparable in effect with haemolysed erythrocytes (Fig. 1(b)), and also with the 'haemoglobin' of BISHOP and GILCHRIST (1946), but less with the 'haemoglobin' of DAY (1954). Conversely, the effect of the writer's haemoglobin or globin approaches that of DAY's 'haemoglobin'. Presumably the writer's stroma, which is no more effective than the genuine haemoglobin, may be also comparable with DAY's 'stroma'. Unfortunately the full data are not available from DAY's report, and BISHOP and GILCHRIST (1946) have not examined stroma. Throughout the comparison, there are extensive fluctuations prevailing

in the ratios of midguts filled. It follows that discordance about the stimulative power of the erythrocyte components might exist, not in the experimental results themselves, but in their interpretation by the different authors. All the facts mentioned above, however, would be reconciled if the stimulus afforded by erythrocytes is essentially chemical and that it passes out gradually into the solvents with which the cell components thus far tested are prepared.

4. *Particulate substances*

Solid particles. Recent authors are in agreement in recognizing that suspensions of particles more rigid than living cells, e.g. starch granules, are scarcely ingested by *Aedes aegypti* (BISHOP and GILCHRIST, 1946; FISK, 1950; DAY, 1954). According to HOSOI (1954a), such mixtures can be imbibed on occasions when they contain sugar as well, but the suspended particles are mostly disposed of and seldom enter the digestive organs. Further evidence is obtained with the following material: (1) starch granules of 10–40 μ in diameter, (2) washed commercial dried brewer's yeast (Ebios) of 3–6 μ in diameter, (3) denatured casein or coagulated haemoglobin, and (4) adsorbent charcoal.

VOL. 3 1959

These particles were suspended in Ringer or egg-emulsion containing 5 per cent glucose and offered by the voluntary technique. Altogether 354 females were exposed, out of which 219 fed nearly to repletion. It was exclusively the crop that was fully distended, in which, nevertheless, none or only traces of the particles could be detected. The midgut was always poorly loaded and also had clear fluid.

Living cells other than erythrocytes. Compared with solid particles, living cells are more readily ingested and drawn into the midgut, as noted by HOSOI (1954a) with a flagellate, and by Day (1954) with sarcosomes of the blowfly. For this reason besides others, the latter author was led to the particle theory of blood stimulation. Since the above results were not satisfactory enough to become unequivocal, further experiments were performed with other materials, i.e. *Euglena* and the ascites tumour cells.

A strain of *Euglena sp.* was supplied from the Botanical Institute of Tokyo University and cultured in Hunter's medium.* After centrifuging off the medium, the organisms were suspended in ox plasma, 0·15 M NaCl, or again in a small amount of the culture medium to be fed forcibly to the mosquito without surplus sugar. In the culture medium the cells took an elongated form about 50–60 μ long and 10 μ wide; when sustaining themselves in saline or plasma, they took a cystoid form of about 15μ in diameter. As shown in Table 1, the cell suspensions in plasma or saline were frequently gorged and sometimes filled the midgut. A comparatively smaller number of the cells, however, was detected in the content of the midgut than in the liquid offered; whereas the concentration of the cells was lowest in the crop, even when this organ was fully distended. In a word,

* The strain of *Euglena* was supplied through the courtesy of Dr. HIROSHI FUJISHIGE of Tokyo University.

TABLE 1—RESULTS OF FORCIBLE FEEDING ON SUSPENSIONS OF *Euglena* AND EHRLICH ASCITES TUMOUR CELLS

Experiments	Liquids offered			Numbers of mosquitoes		
	Main constituents	Ratios of cells	Glucose content (%)	Exposed	Satiate	Midgut-filled
A1	*Euglena* in ox plasma	1/10	0	12	5	1
	Ox plasma	0	0	9	5	0
	Ox erythrocytes in plasma	1/2	0	12	6	6
A2	*Euglena* in ox plasma	1/2	0	5	3	2
	Euglena in 0·15 M NaCl	1/2	0	5	2	1
	Euglena in culture medium	4/5	0	5	0	0
	Ox plasma	0	0	5	0	0
	Ox erythrocytes in plasma	1/2	0	5	5	5
B1	Tumour cells in ascites	1/4	0	6	5	5
	Tumour cells in ascites	1/2	0	11	9	9
	Ascitic fluid	0	0	17	2	0
	Ox whole blood	11/20	0	17	10	10
B2	Tumour cells in ascites	1/2	2	11	7	7
	Ascitic fluid	0	2	11	6	0
	Ox whole blood	11/20	2	11	8	8

Euglena cells exerted a certain degree of the blood-like stimulation. It will be considered later why their suspension in the culture medium failed to cause satiation (Section B, 3).

Similar examinations were made of Ehrlich ascites tumour cells.* Mouse ascites containing $\frac{1}{4}$ the volume of the tumour cells (about 20 μ in diameter) were offered to the mosquito, either immediately or after being doubled in cell concentration by centrifuging, and also either with or without addition of 2 per cent glucose. The results were remarkable (Table 1). The tumour cells suspended in the ascites proved as effective as ox whole blood in filling the midgut even in the presence of glucose. In contrast, the ascitic fluid alone was no more stimulating than ox blood plasma. Since the ascites contained no appreciable amount of haemoglobin, the results would bear out the view that the stimulative power of

* They were kindly supplied for use by Dr. TADASHI YAMAMOTO of the Institute for Infectious Diseases, Tokyo University.

erythrocytes is by no means concerned with their large content of haemoglobin. This conclusion, however, does not necessarily favour the particle theory; it favours rather the prediction that a certain substance other than haemoglobin stimulates the mosquito (Section A, 3). The only modification requisite to the latter opinion is that the substance need not be solely contained in erythrocytes, but it may be widely distributed over various kinds of living cells.

5. *Negative results with miscellaneous substances*

Several feeding tests were carried out at times with substances which were suspected of having a blood-like stimulus. All the following liquids failed to sate the mosquito, whereas addition of glucose invariably rendered them acceptable to the insect, with the consequent distension of the crop: (1) an emulsion of hen's egg slightly diluted with Ringer or distilled water; (2) 1–5 per cent solutions of casein in 0·1 M $NaHCO_3$; (3) a 2 per cent solution of histidine in ox blood plasma; (4) 1–2 per cent solutions of sodium glutamate in 0·15 M NaCl; and (5) 0·15 M NaCl containing 10^{-4}–10^{-6} mg/ml of adrenalin, acetylcholine, or histamine. Similar negative results are also recorded by Fisk (1950) on peptone and amino acids.

VOL. 3 1959

B. *Factors Regulating the Action of Sugar and Blood*

1. *Concentration*

Thresholds of gorging for sugars. So far as mosquitoes are concerned, very few records have appeared of thresholds of any kind for contact chemoreception. Since it was frequently required to set up a standard for sugar acceptance of the test animals, an attempt was made to determine concentrations of glucose, sucrose, and maltose just sufficient to induce gorging of *Culex pipiens* var. *pallens* females. These critical concentrations, therefore, should be naturally higher than those for mere acceptance or detection of the sugars. The test compounds were dissolved in distilled water so as to provide a series of doubling concentrations and offered to 2–3-day-old mosquitoes not fed previously on any sugar, though not necessarily water-sated. As shown in Table 2, glucose brings about engorgement of some individuals at $\frac{1}{8}$ M; whereas the critical concentrations for both sucrose and maltose are roughly $\frac{1}{16}$ to $\frac{1}{32}$ M. The values for these two sugars are not significantly different. Hence the order of acceptance for the three sugars is sucrose = maltose > glucose, thus following a trend similar to those confirmed with other insects (Minnich, 1929, 1931; von Frisch, 1934; Haslinger, 1935; Schmidt, 1938; Hassett, Dethier, and Gans, 1950).

Thresholds of gorging for erythrocytes and their components. The concentrations of ox erythrocytes and their components effectively inducing satiation were determined with their serially diluted suspensions or solutions. In the case of intact erythrocytes the diluent was either plasma or 0·15 M NaCl; in other cases only the saline was utilized. Haemolysis was accomplished by addition of a minimum amount of water to washed erythrocytes, with subsequent addition of a requisite amount of NaCl to yield 0·15 M solution with respect to the salt. For separating

stroma and endosoma, erythrocytes were laked in 7 times the volume of 0·01 N HCl, and the resulting mixture was centrifuged. The supernatant was regarded

TABLE 2—THRESHOLDS OF GORGING FOR GLUCOSE, SUCROSE AND MALTOSE DETERMINED BY THE FORCIBLE TECHNIQUE OF FEEDING

Experiments	Molar concentrations of sugars	Numbers of mosquitoes fed*		
		Glucose	Sucrose	Maltose
A	1/2	7-0-0	7-0-0	7-0-0
	1/4	6-0-1	7-0-0	7-0-0
	1/8	2-4-1	7-0-0	7-0-0
	1/16	0-2-5	6-1-0	7-0-0
	1/32	0-1-6	1-2-4	6-1-0
	1/64	0-0-7	0-1-6	1-1-5
	1/128	—	—	0-0-7
B	1/4	3-3-1	—	—
	1/8	1-3-3	7-0-0	—
	1/16	0-2-5	6-1-0	6-1-0
	1/32	—	2-4-1	0-1-6
C	1/8		5-0-0	4-0-1
	1/16		1-1-3	0-3-2
	1/32		0-2-3	0-0-5

* Three figures joined by hyphens denote from the left to the right, respectively, the numbers satiate, partially fed, and fed slightly or refused. In A, the animal sources are different for dissimilar sugars.

as an 8-fold dilute solution of endosoma, of which the NaCl content was again adjusted. The sediments, after being washed twice with 0·15 M NaCl, were

TABLE 3—THRESHOLDS OF GORGING FOR OX ERYTHROCYTES AND THEIR COMPONENTS DETERMINED BY THE FORCIBLE TECHNIQUE OF FEEDING ON THE SUGAR-FREE SUSPENSIONS

Volume ratios of cells or components	Numbers of mosquitoes fed*					
	Intact cells in plasma	Intact cells in saline	Laked cells in saline	Stroma in saline	Endosoma in saline	Stroma and endosoma in saline
1/2	—	—	—	1-7-2	—	—
1/4	9-2-1	7-1-0	11-0-4	1-3-6	—	—
1/8	6-2-4	6-0-2	13-0-2	3-4-3	4-5-1	—
1/16	1-4-7	4-4-0	9-3-3	1-5-4	5-2-3	2-3-0
1/32	2-3-7	3-4-1	10-1-4	0-2-8	0-4-6	2-2-1
1/64	1-4-7	2-2-4	2-4-9	—	—	1-3-1
1/128	0-2-10	0-2-6	0-4-11	—	—	—

* Three figures joined by hyphens denote from the left to the right, respectively, the numbers satiate, partially fed, and fed slightly or refused.

suspended in an equal part of the saline, thus ensuring a 2-fold dilution of stroma. In case this was likely to precipitate, a small amount of NaOH was added to restore neutrality. The results are summarized in Table 3. On the whole, the erythrocytes, either intact or laked, proved effective down to concentrations of $\frac{1}{32}$ to $\frac{1}{64}$ in terms of the cell volume. The efficiency of the combined stroma and endosoma seemed more than a mere sum of the action of the two components.

2. *Chemical influences*

Experiment 1. Among the several experiments purposed for outlining the influences of various compounds upon sugar or blood feeding, a preliminary comparison was made of the following solutions: (a) NaCl, NH_4Cl, $CaCl_2$, KCl, KNO_3, K_2SO_4, $MgSO_4$, HCl, NaOH, urea, ethanol, glycerol, and glucose, all in aqueous solutions isotonic with 0·15 M NaCl; and (b) 0·005 M quinine hydrochloride in 0·15 M NaCl. As a base, 0·5 M glucose and an 8-fold dilute aqueous suspension of haemolysed erythrocytes were utilized. As shown in Table 4, solutions of certain inorganic chlorides (here NaCl and $CaCl_2$) were preferred

VOL. 3 1959

TABLE 4—ACCEPTANCE AND REJECTION OF VARIOUS COMPOUNDS EXAMINED BY THE FORCIBLE FEEDING ON THE LIQUIDS CONTAINING EITHER GLUCOSE OR HAEMOLYSED ERYTHROCYTES OR NOT CONTAINING THESE

Test liquids	Numbers of mosquitoes exposed (E), satiate (S), and midgut-filled (M)								
	Test liquids alone			Test liquids +glucose*			Test liquids +erythrocytes†		
	E	S	M	E	S	M	E	S	M
Distilled water	5	0	0	3	3	0	4	0	0(!)
Ringer	5	0	0	—			—		
0·15 M NaCl	5	1	1	3	3	0	4	3	3
0·15 M NH_4Cl	5	0	0	3	3	0	4	1	1
0·1 M $CaCl_2$	5	1	0	3	3	0	4	3	3
0·15 M KCl	5	0	0	3	3	0	4	4	4
0·15 M KNO_3	5	0	0	3	3	0	4	3	3
0·1 M K_2SO_4	5	0	0	3	3	0	4	2	2
0·15 M $MgSO_4$	—			3	3	0	4	2	2
0·15 M HCl	5	0	0	—			4	0	0
0·15 M NaOH	5	0	0	—			4	1	0
0·3 M urea	5	0	0	3	3	0	4	4	4
0·3 M ethanol	5	0	0	3	3	0	4	1	0
0·3 M glycerol	5	0	0	3	3	0	4	0	0
0·3 M glucose	5	4	0	—			6	5	2(!)
0·005 M quinine (a)	—			3	0	0	5	0	0
Ox plasma	—			3	3	0	—		
Ox erythrocytes (b)	—			3	3	0(!)	—		

* Yielding 0·5 M solutions with respect to glucose.
† Containing $\frac{1}{8}$ the total volume of laked erythrocytes.
(a) Quinine hydrochloride in 0·15 M NaCl.
(b) Containing $\frac{1}{8}$ the total volume of laked erythrocytes in 0·15 M NaCl.

slightly to distilled water. This result is in conformity with the previous result (Section A, 1). Generally speaking, however, the insect was rather indifferent to all the seven inorganic salts inclusive of $MgSO_4$, at least in such concentrations, and so it was to urea. Quinine was evidently rejected; HCl and NaOH also rendered the blood unacceptable. Apparently ethanol and glycerol in the given concentrations did not affect sugar feeding, though they definitely hindered blood feeding. Probably the difference was derived from the greater acceptability of 0·5 M glucose as compared with the diluted erythrocytes. It was impossible, however, to account by analogy for the unambiguous deterioration which occurred in the base blood containing no additional compound except water. This paradoxical phenomenon will be considered later (Section B, 3). Another problem of dispute concerned the destination of blood. The marked rises in the proportion of crop-filling with the present blood-sugar mixtures as compared with the previous result (Section A, 2) could be explained by the differences in the relative concentrations of erythrocytes and glucose. This also necessitated a more comprehensive experiment (Section B, 4).

Experiment 2. To supplement experiment 1, the rejection thresholds of gorging for HCl, NaOH, and quinine hydrochloride were roughly estimated employing 0·5 M glucose as the base. The sugar meal was definitely refused in the presence of 0·1 M HCl or NaOH, but was accepted without much hindrance when containing these at 0·01 M. In contrast, rejection of quinine was consistent at 0·001 M and still perceptible at 0·0001 M.

Experiment 3. In a similar experiment, but utilizing 0·25 M sucrose as the base instead of 0·5 M glucose, the rejection thresholds of gorging for $ZnCl_2$, $FeCl_3$, $FeSO_4$, and $CuCl_2$ were found to be between 0·1–0·01 M, 0·075–0·0075 M, 0·15–0·015 M, and 0·001–0·0001 M, respectively, thus demonstrating the exceeding sensitivity of the mosquito to Cu^{++} ions. The outstanding effect of Cu^{++} agrees with the prediction by FRINGS (1946).

Experiment 4. Sugar feeding thus far examined seems apparently uninfluenced by the presence of 0·15 M NaCl. It was confirmed, however, that higher concentrations of the salt definitely reduced the percentage acceptance of sugar solutions. Occasionally such an effect of NaCl was observed even at a concentration of 0·15 M. As shown in Table 5,

TABLE 5—INFLUENCE OF NaCl AND UREA UPON THE FORCIBLE FEEDING ON GLUCOSE SOLUTIONS

Molar concentrations of glucose	Numbers of mosquitoes fed*				
	Glucose in distilled water		Glucose in 0·15 M NaCl		Glucose in 0·3 M urea
	Warm	Cold	Warm	Cold	Warm
1	4-1-0	4-1-0	1-2-2	2-3-0	5-0-0
1/2	4-0-1	3-1-1	1-3-1	1-4-0	3-1-1
1/4	1-3-1	1-3-1	1-2-2	2-1-2	1-2-2
1/8	1-1-3	0-2-3	0-1-4	0-2-3	0-0-5
1/16	2-2-1	0-1-4	1-4-0	1-1-3	1-0-4
1/32	0-3-2	0-0-5	0-4-1	2-2-1	0-0-5
0	0-3-2	0-1-4	0-2-3	1-4-0	0-1-4

* Three figures joined by hyphens denote from the left to the right, respectively, the numbers satiate, partially fed, and fed slightly or refused.

deterioration of the serially diluted glucose solutions by NaCl is manifest for higher concentrations of the sugar, making a contrast to the influence of urea. With respect to lower

concentrations of the sugar, the presence of 0·15 M NaCl becomes again favourable to feeding as observed previously. The nature of this dual influence of NaCl is obscure at present; but the phenomenon may tentatively be ascribed to the double modality of this kind of salt (DETHIER and CHADWICK, 1948).

Experiment 5. The effect of hydrogen-ion concentration on blood feeding was examined by feeding the mosquito forcibly on sugarless suspensions of ox erythrocytes, stroma, and endosoma, all in 8-fold dilution in 0·15 M NaCl except stroma, which was diluted to 4-fold as well as 8-fold. The pHs were adjusted with HCl and NaOH to several values between 3·5 and 10·5 as determined with the aid of a glass electrode pH-meter. Both haemolysed erythrocytes and endosoma proved effective over the entire range of the above pH, though their efficiency was reduced more or less extensively on the alkaline side. The stroma sedimented at a lower pH rendered the suspension quite unacceptable.

3. *Temperature, viscosity, and osmotic pressure*

Temperature. According to BISHOP and GILCHRIST (1946), blood feeding of *Aedes aegypti* is promoted by a heat gradient, though there is also an optimum temperature for that activity. The writer frequently found that even under forced conditions *Culex pipiens* var. *pallens* could hardly gorge on shed blood when its temperature fell below 15°C or so. It is reported, however, that neither tarsal stimulation of the butterfly (WEIS, 1930) nor sugar feeding of honeybees (VON FRISCH, 1934) is seriously influenced by temperatures over a wide range. This seems also to hold true in sugar feeding of the mosquito. In an experiment described previously (Section B, 2, experiment 4; Table 5), some series of the test solutions were warmed to initial temperatures of 45–50°C, whereas the others were applied at 10–13°C. Although the actual difference in the feeding temperatures could not be so extensive as it appeared to be, no evidence was obtained to contradict the above proposition.

Influence of viscosity and osmotic pressure on sugar feeding. Previous authors are in accord in recognizing that the stimulative power of various compounds in contact chemoreception of insects, either in acceptance or rejection, is not dependent upon viscosity or upon osmotic pressure (MINNICH, 1921, 1929; WEIS, 1930; VON FRISCH, 1934; DETHIER and CHADWICK, 1947). It was reported also that concentrated solutions of glucose in Ringer were gorged by the mosquito with consistent filling of the crop regardless of the viscosity (HOSOI, 1954a). In this instance the most viscid solution, of which the relative viscosity measured 12·5, was 4·6 M, or an 83 per cent solution with respect to glucose. The sole change occurring in feeding on this solution was protraction of the aspiration period from a usual duration of about 1 min to more than two. Therefore, both viscosity and osmotic pressure of sugar solutions had no fundamental influence upon the sequence of feeding.

Influence of osmotic pressure on blood feeding. In spite of the above fact there are also reasons to suppose that osmotic pressure probably affects the feeding response of the mosquito at least on sugarless liquids which are destined to pass primarily to the midgut; i.e. (1) salines isotonic with blood are preferred to distilled water (Sections A, 1; B, 2); (2) a suspension of *Euglena* in ox blood plasma or in

0·15 M NaCl is gorged, whereas that in the culture medium is rejected (Section A, 4); and (3) ox erythrocytes laked in excess distilled water are rejected unless the osmotic pressure is adjusted (Section B, 2). After all, osmotic pressure might influence the mosquito in such a way as to impede its normal act of blood ingestion, and not in a way to simulate the action of blood, as FISK (1950) failed to note. It seems likely that some of the failures of, or difficulties found in, feeding mosquitoes on blood components by previous workers (BISHOP and GILCHRIST, 1946; DAY, 1954; HOSOI, 1954a) are also caused by this overlooked effect of osmotic pressure on blood ingestion. An experiment was undertaken to test the above prediction.

Test animals were fed forcibly and exclusively on laked ox erythrocytes in 8-fold dilute solutions, of which the osmotic pressure had been adjusted by adding one of the following compounds: glucose, sucrose, urea, NaCl, and a combination of NaCl, KCl, and $CaCl_2$ in proportions as in Ringer. By

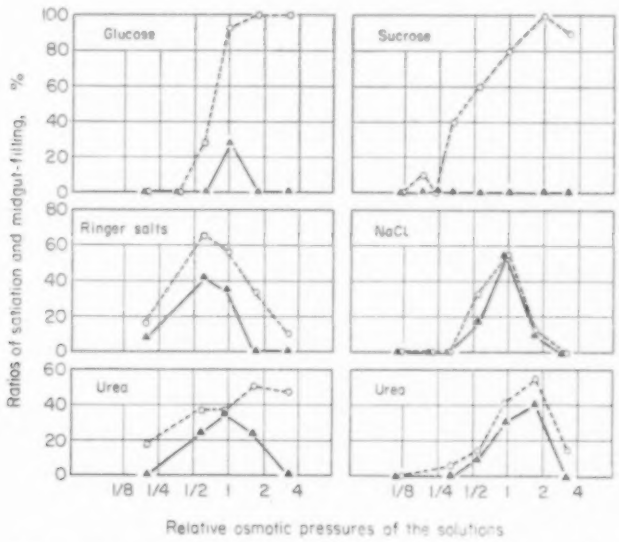


FIG. 3. Effects of osmotic pressure upon gorging of the mosquitoes forcibly fed on haemolysed erythrocytes.

Trends of percentages satiate/exposed and those midgut-filled/exposed are drawn, respectively, by broken and solid lines. Each liquid contains $\frac{1}{4}$ the total volume of laked erythrocytes and its osmotic pressure is adjusted by the compound designated in the figure. The relative osmotic pressures are represented in terms of multiples of the pressure equivalent to that of 0·15 M NaCl.

mixing a solution with another which contained the same kind of the solute at a different concentration, five series of erythrocyte solutions were obtained, all the solutions being identical with respect to the concentration of blood, but each differing from the others either concerning the osmotic pressure or the

kind of the solute whereby the pressure was maintained. To each solution 10–12 mosquitoes were subjected. In estimating the feeding activity, the numbers of fully engorged mosquitoes were weighted at 1 and those of the partially filled at 0·5. The values were summed for each solution and represented as percentages. Similar calculations were also made of the numbers of full midguts. The results are illustrated in Fig. 3, in which all the values for solutions isotonic with 0·15 M NaCl, and so also isotonic with blood of terrestrial vertebrates, are plotted on the ordinates denoted 1.

Evidently solutions of erythrocytes in salines reduce their effectiveness concerning both gorging and midgut filling when the osmotic pressure is extensively diverted from the normal blood value. A similar trend is perceptible with solutions in which urea maintains the pressure instead of inorganic salts, although the urea solution seems most effective at a slightly hypertonic region. In contrast, feeding activity on the sweetened erythrocyte solutions rises with the increasing content of the sugars until it attains the maximum height for 0·6 M glucose or sucrose, i.e. double the osmotic pressure of normal blood; thereafter the activity is maintained nearly constant. In this instance an important point is that midgut-filling does not follow this trend. It takes place only and slightly in the solution containing 0·3 M glucose, but not in 0·3 M sucrose which is to taste sweeter than glucose (Section B, 1). Under these circumstances the effect of blood is undoubtedly masked by that of the sugars.

Regarding the influence of the test compounds in higher concentrations, whether promotive or inhibitory to blood feeding, it is not necessary to resort to the action of osmotic pressure. However, the consistent reduction of the effectiveness towards the hypotonic side in all kinds of solutions can hardly be explained otherwise than by postulating that inadequate osmotic pressure impedes the feeding response in some way.

4. *Competitive action of sugar and blood*

It seems likely that discordant results obtained by earlier workers (MacGregor and Lee, 1929; MacGregor, 1931; Roy and Ghosh, 1940; Bishop and Gilchrist, 1946; Trembley, 1952) on the distribution of blood-honey or blood-sugar mixtures in the digestive organs of mosquitoes are in part derived from the difference in concentrations of the two components in the mixtures used. Day (1954) fed *Aedes aegypti* on mixtures of blood and sugars in various proportions, pointing out that the relative concentration of the components of a mixture determines its destination. The presence of a similar fact in *Culex pipiens* var. *pallens* has already been suggested (Section B, 2, experiment 1). An improved experiment was set up in which the mosquito was fed forcibly on mixtures of haemolysed ox erythrocytes and sucrose.

The mixtures were made isotonic with normal blood or with 0·15 M NaCl, and were made in four series which contained erythrocytes in $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, and $\frac{1}{32}$ proportions to the total volume. The above mixtures could be arranged again in four series with respect to the concentrations of sucrose which were 0·15 M,

0·075 M, 0·038 M, and 0·019 M, respectively. Thus the arrangement was to provide 16 solutions, of which 10 could actually be examined under an identical condition. Since the amounts of the sugar fell short of maintaining the desired osmotic pressure, the shortages were covered by addition of NaCl.

Five mosquitoes were subjected to each solution, and the amount of the liquid ingested by each animal as well as that found in the crop and midgut was grouped according to conveniently set-up grades. These grades had been evaluated by actually weighing similarly fed individuals with the aid of a torsion balance, so that weighting the number of the animals or organ belonging to each grade at the corresponding factor and summing up the products for each solution gave approximately a relative value for the total quantity of the liquid taken up by a lot or found in either the crop and midgut. Fig. 4 represents the results.

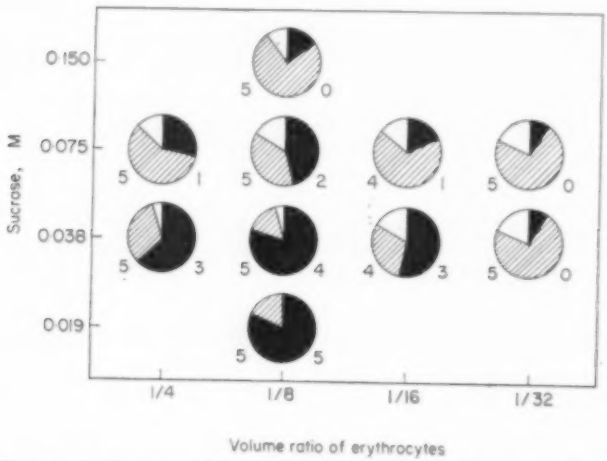


FIG. 4. Changes in the proportion of midgut-filling depending upon relative concentrations of sucrose and haemolysed erythrocytes in isotonic solutions.

Each circle accompanied by a pair of figures represents the result of forcible feeding of 5 mosquitoes on a single solution, where the number satiated is shown on the left and that of the midgut-filled on the right. A sum proportion of the midgut contents is represented by a solid area in the circle and that of the crop contents by a shaded one, the combined areas providing a measure of acceptance.

All 50 mosquitoes except two gorged fully or fed nearly to repletion. For any given concentration of erythrocytes, both the number of full midguts and the total amount of the food loading of the midgut increased with the decreasing content of sucrose. For a given concentration of sucrose, the above number and amount increased with the increasing content of erythrocytes, attaining a maximum for an 8-fold dilution of the cells. On the whole, the effect of erythrocytes became hardly discernible at 32-fold dilution and that of sucrose hardly discernible at the concentration of 0·019 M. These critical concentrations approximate those confirmed previously with separate solutions of sugar and the blood (Section B,

1). There was no sign that the efficiency of feeding was reduced on account of the co-existence of blood and sugar in the food liquid.

On the other hand, examination of the food distribution with respect to each individual disclosed the fact that in few mosquitoes was the imbibed blood-sugar mixture shared equally between the crop and midgut. Out of 48 engorged, 17 exhibited fully distended midguts; among these, 12 showed no more than a trace of the mixture in the crop. Conversely, 29 out of the above 48 had fully distended crops; among these, 14 showed no more than a trace of food in the midgut. In all, only two showed the crop and midgut equally distended. The result would suggest that a fairly slight difference in the stimulative power of sugar and blood is appreciated by the mosquito and the insect tends to disregard the minor stimulus. In other words, central selection would probably operate in this circumstance.

C. *Identification of the Blood Stimulant*

Routine method of tests. Assuming that the stimulating principle of blood cells is a substance (Section A, 3 and 4), then it may be extractable from the cells by the use of some suitable solvents. Extraction was certainly realized at one time or another with dilute ethanol; nevertheless, success could by no means be consistently obtained in later experiments. By far the greatest hindrance encountered during the earlier efforts was the misleading negative results probably due to inadequate preparation of the test liquids. There were many chances of introducing or accumulating repugnant compounds in the extract, and the proper adjustment of osmotic pressure was not accomplished with certainty. Since no means could be devised to deal safely with such hazards, the following method was conveniently adopted in routine tests.

Original samples of the extracts were mixed with 0·15 M NaCl in series of 5-fold dilute concentrations, and each of the resulting solutions were fed forcibly to 5–7 females without addition of sugar. Occurrence of rapid gorging with consequent filling of the midgut was considered to be a positive response. A solution was regarded as effective when it produced two or more positive responses. The effectiveness of an extract was judged both by the number of the positive responses and by the order of the effective dilution.

Extraction of the acceptable substance. Citrated ox blood furnished the material almost exclusively. After a large number of trials, the following procedure was established as the most reliable, if not the most efficient, way of obtaining consistent results:

The blood was centrifuged to remove plasma, and the sedimented cells were washed once with 0·15 M NaCl. Two parts of the cells were poured into a mixture of 1 : 6 : 1 parts respectively of water, ethanol, and ether. After standing, the mixture was filtered, and the clear filtrate was combined with first cold and then hot water washings of the residue mostly consisting of coagulated haemoglobin, which was finally discarded. Condensation of the solution followed by evaporation at 60°C under a reduced pressure yielded a small amount of an ochre coloured residue which was partially soluble in distilled water. This residue, when emulsified in the original volume of water, proved slightly less effective than intact erythrocytes; nevertheless, its activity per unit dry weight was estimated as being several times as high as that of erythrocytes. It withstood at least a

few hours' boiling in neutral water and several months' desiccation with $CaCl_2$. It was confirmed that similar substances were also extractable from the liver, kidney, ovary, spleen, and brain of the rabbit but not from ox blood plasma.

The dried extract of ox blood cells was then mixed with a small amount of distilled water and dialysed against distilled water which was usually boiling. Although a larger proportion of the activity remained persistently with undialysable substances, these were not treated further on account of the difficulty of separating the components. Notwithstanding the inevitable loss which ensued from the treatment, there was a marked rise in the activity of the dialysate per unit dry weight. Since the effective substance proved insoluble in fat solvents but readily soluble in water, it was impossible by the use of such solvents to remove excess inorganic salts and a certain yellowish pigment also contained in the dialysate. The effective substance could be adsorbed on charcoal in a slightly acidic cold aqueous solution and subsequently released into a hot solution of dilute ammonia which was easily removable afterwards. It was also soluble in slightly acidic 95 per cent ethanol, from which it precipitated when the solvent was neutralized. Finally, the use of ion-exchange resin, e.g. sorption on Amberlite IRA-410 (OH-form) and subsequent elution with 0·01 N HCl, provided more satisfactory means of desalting and depigmentation. On the other hand, sorption on a strongly acidic cation exchanger brought about deterioration of the effective substance.

Paper chromatographic separation of the extract. The extract obtained through the above procedure still contained an appreciable amount of yellowish pigment as well as many amino acids. A unidimensional paper chromatogram of the extract developed by the ascending method demonstrated that, when water-saturated n-butanol containing acetic acid was used as the solvent, the R_F value of the effective substance should lie between 0·1 and 0·3, and that the spot overlapped on some of the spots of amino acids such as glutamic, aspartic, cystine, histidine, arginine, lysine, and ornithine. The effective substance remained on the base line when the development was performed with water-saturated n-butanol containing ammonia. At any rate, paper chromatographic separation of the extract was not very successful owing to the severe restriction in the use of suitable solvents, which were required to be least repugnant to the mosquito because contamination of them in the test liquids invalidated the feeding experiments.

Separation by ion-exchange chromatography. All the facts thus far known about the nature of the effective substance indicate that it belongs to an ampholite of a relatively small molecular size. Since amino acids are unlikely to stimulate the mosquito in such a characteristic way as found in blood feeding (Section A, 5), nucleotides come naturally to mind. Hence, an attempt was made to separate the components of the extract by the technique of ion-exchange chromatography which was designed by COHN (1950) for separation of ribonucleotides, with a difference that Amberlite IRA-410 (Cl-form) was tentatively utilized as the exchanger instead of Dowex-2-chloride. Fractions of the eluate were neutralized with NaOH, evaporated, re-dissolved in requisite amounts of distilled water

sufficient to yield 0·15 M solutions with respect to NaCl, and finally diluted with 0·15 M NaCl to a definite volume. The results of the feeding tests are represented in Fig. 5. Most of the positive responses appeared in fractions which were later found to contain adenylic acid. These fractions, however, still contained a small amount of the yellowish pigment as well as traces of amino acids detectable on a paper chromatogram.

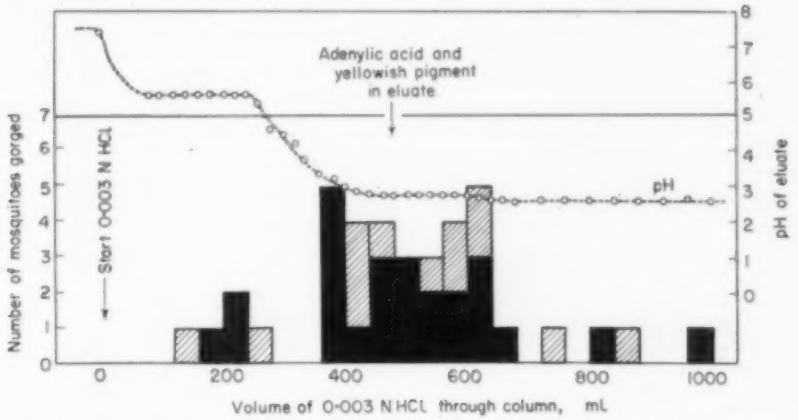


FIG. 5. Detection of the stimulative compounds in fractions of an ox erythrocyte extract separated by anion-exchange chromatography.

Exchanger: Amberlite IRA-410 (Cl-form), 100–200 mesh, 19 cm × 0·8 cm². Sorbed material: partially purified dialysate of an ox erythrocyte extract obtained from 400 ml of the fresh cells. Elution: with 0·003 N HCl. Test: by forcible feeding on neutralized, 4-fold condensed and osmotically adjusted fractions of the eluate. Numbers satiate are shown by solid columns and those partially fed by shaded ones.

Identification of the stimulative compound. In order to eliminate the sources of above-mentioned contaminations, yeast pentose nucleic acid (Merck manufacture, Na-salt) was hydrolysed by the method of CHARGAFF *et al.* (1950), and the hydrolysates were separated likewise as described above. Against expectation, all the fractions of the eluate were found virtually ineffective except that one or two sporadic cases of positive response took place. The original solution of the hydrolysates also proved negative.

Finally, the effective fractions of the resin eluate referred to in Fig. 5 were combined together, treated with adsorbent charcoal to remove excess pigment, and subjected to spectroscopic examination with the aid of a Hitachi EPU-II spectrophotometer. At the same time another preparation treated similarly but obtained from a different source was examined. For both preparations the results gave unambiguous ultra-violet absorption spectra of adenylic acid, with maximum extinctions at 259 mμ at pH 5·4. As to the former solution the absorbence ratios at pH 5·4 for A_{250}/A_{260}, A_{280}/A_{260}, and A_{290}/A_{260} were 0·822, 0·206, and 0·063, respectively; whereas in the latter these values were respectively 0·820, 0·312,

and 0·232. From computation, the original dialysates of the extracts obtained from 1000 ml of ox erythrocytes were estimated to contain on the average $3{\cdot}5 \times 10^{-4}$ mole of adenylic acid of which 10^{-4} to 10^{-5} M solutions could sufficiently stimulate the mosquito to cause full feeding on them. This finding was immediately confirmed with a preparation of C.P. grade of muscle adenylic acid, which, being dissolved in 0·15 M NaCl, proved highly effective at a concentration of 0·0001 M.

D. *Comparative Studies with Nucleotides and Related Compounds*

Quality of the compounds examined. The discordant results obtained with the extracts of ox blood cells as well as muscle adenylic acid on one hand, and with the hydrolysates of yeast pentose nucleic acid on the other, necessitated comparisons of effectiveness among various compounds related to the nucleotide under conditions as equal as possible. Altogether 16 such compounds besides six amino acids were available for the present purposes.

(1–3) D-ribose, adenine and adenosine: prepared at the Laboratory of Organic Chemistry, Tokyo Institute of Technology; highly pure.

(4) Theophylline ribose-5-phosphate (7-β-D-ribofuranosyltheophylline monophosphoric acid): synthesized at the above laboratory.

(5) 5′-adenylic acid (muscle adenylic or adenosine-5′-monophosphoric acid): Schwarz Biochemical Inc. manufacture, presented by Dr. T. SATO of the above laboratory with his guarantee.

(6) 2′ or 3′-adenylic acid (yeast adenylic or adenosine-2′ or 3′-monophosphoric acid): Nutritional Biochemical Corp. manufacture, of uncertificated purity.

(7) Riboflavin-5′-phosphate (the Na-salt): Light and Co. manufacture, of uncertificated purity.

(8) 5′-guanylic acid (Na-salt of guanosine-5′-monophosphoric acid): Pabst Brewing Co. manufacture, of C.P. grade.

(9–11) 5′-deoxyadenylic acid (deoxyadenosine-5′-monophosphoric acid), 5′-deoxyguanylic acid (NH_4-salt of deoxyguanosine-5′-monophosphoric acid) and 5′-thymidylic acid (Ca-salt of thymine-deoxyriboside-5′-monophosphoric acid): California Foundation for Biochemical Research manufactures, guaranteed with data for ultraviolet extinctions.

(12) Adenosine-5′-sulphate (Ba-salt of adenosine-5′-monosulphuric acid): synthesized by Professor F. EGAMI at Nagoya University (EGAMI and TAKAHASHI, 1955) and sent by him.

(13) Ribose-5-phosphate (the Ba-salt): Nutritional Biochemical Corp. manufacture, containing *ca.* 5 per cent (mole) adenylic acid; after purification the contamination fell to 1·0 per cent.

(14) 5′-inosinic acid (Ba-salt of inosine-5′-monophosphoric acid): converted from 5′-adenylic acid by the writer and its purity proved spectrophotometrically.

(15) Adenosine triphosphate (Ba-salt of adenosine-5′-triphosphoric acid): Zellstoffabrik Waldhof manufacture. The original sample contained 14·3 per cent, 23·4 per cent and 62·2 per cent (mole) respectively of adenosine mono-, di- and tri-phosphate according to ion-exchange chromatographic analysis kindly performed by Dr. SHIGETOSHI TAGUCHI of Tokyo University. The test solutions were obtained from the eluate of the sample sorbed on Amberlite IRA-410 (Cl-form). The purities will be referred to later.

(16) Adenosine diphosphate (adenosine-5′-diphosphoric acid): the least contaminated preparation was obtained as a by-product during the purification procedure of adenosine triphosphate.

These compounds, previously converted to sodium salts when supplied in the form of free acids or Ba-salt, were dissolved in 0·15 M NaCl, with which 10-fold serial dilutions were performed. The pHs were adjusted to *ca.* 7 with 0·001 M $NaHCO_3$ or with 0·001 M-equivalent Sørensen's phosphate buffer. As a control, similarly buffered 0·15 M NaCl was used. In addition to these, the following amino acids were also examined: glycine, glutamic acid, cysteine, arginine, tyrosine, and histidine. All the compounds were fed forcibly to the mosquito without addition of sugar, usually at the concentrations of 1×10^{-3} M, 1×10^{-4} M, and 1×10^{-5} M, and the condition of the animals was checked by similar feeding tests on 1×10^{-4} M and 1×10^{-5} M 5′-adenylic acid.

Relative effectiveness of the compounds. The results are shown in Tables 6 and 7. With all the efforts to eliminate impurities of the test compounds, there were inevitable sources of possible contaminations which might affect the results more or less extensively. Apart from the fact that commercial preparations of nucleotides contained sometimes considerable amounts of adenylic acid possibly of the 5′-phosphate, adenosine di- and tri-phosphates, unlike the other compounds tested, had been suspected of rapid decomposition under the conditions of

VOL. 3 1959

TABLE 6—A SUMMARY OF THE FORCIBLE FEEDING OF THE MOSQUITOES ON SERIALLY DILUTED SUGARLESS SOLUTIONS OF 5′-ADENYLIC ACID IN 0·15 M NaCl (pH *ca.* 7)

Molar concentrations	Numbers of mosquitoes				Ratios		
	Exposed	Fed			F/E	S/E	P/S
	(E)	Satiate (S)	Partially fed (P)	Total (F)	(%)	(%)	
1×10^{-2}	10	4	1	5	(50)	(40)	(0·25)
1×10^{-3}	50	26	5	31	62	52	0·19
1×10^{-4}	475	233	70	303	64	49	0·30
1×10^{-5}	435	70	57	127	29	16	0·81
1×10^{-6}	50	3	0	3	6	6	(0)
1×10^{-7}	10	0	1	1	(10)	(0)	—

the feeding test. This, however, was not the fact. Ion-exchange chromatographic separation of the test liquids followed by spectrophotometric measurements of the components demonstrated that adenosine triphosphate endured 2 hr warming at 45°C in a solution of pH 7, without significant increase in the amounts of adenosine mono- and di-phosphate. In one of such assays, molar percentages for adenosine, adenosine mono-, di-, and tri-phosphate in a preparation of presumed triphosphate were 0·0, 2·0, 18·6, and 79·4, respectively; immediately after the test the percentages had changed to 0·3, 2·9, 21·9, and 74·9 respectively. It follows that the contaminations detected in the di- and tri-phosphate solutions after the test (Table 7) should have appeared before or during the preparation of the solutions and not during the feeding tests.

TABLE 7—STIMULATIVE POWER OF VARIOUS NUCLEOTIDES AND THEIR RELATED COMPOUNDS TO INDUCE GORGING OF THE MOSQUITO

Compounds	Numbers of mosquitoes exposed (e) and fed (f)*									
	Experimental compounds†						5′-adenylic acid†			
	10^{-3} M		10^{-4} M		10^{-5} M		10^{-4} M		10^{-5} M	
	e	f	e	f	e	f	e	f	e	f
D-ribose	22	0-2	22	0-1	—		20	13-2	20	1-2
Adenine	22	0-1	22	1-0	—		20	13-2	20	1-2
Adenosine	39	2-5	39	2-1	14	0-0	20	12-1	20	4-3
Ribose-5-phosphate (a)	20	1-0	20	0-0	—		20	13-4	20	3-4
2′ or 3′-adenylic acid	45	3-5	45	1-3	45	0-1	35	15-3	35	6-3
Adenosine diphosphate (b)	—		50	25-6	50	13-9	50	22-8	50	10-9
Adenosine triphosphate (c)	—		50	18-6	50	6-9	50	22-8	50	10-9
5′-inosinic acid	20	0-0	20	0-0	20	0-0	20	10-4	20	2-2
5′-guanylic acid	20	0-3	20	0-1	20	1-1	20	9-2	20	4-4
Theophylline ribose-5-phosphate	22	0-1	22	0-1	22	0-0	20	13-3	15	1-2
5′-deoxyadenylic acid	20	12-3	40	4-9	40	1-5	20	9-2	20	4-4
5′-deoxyguanylic acid	20	3-1	20	1-1	—		20	10-4	20	2-5
5′-thymidylic acid	20	0-5	20	0-5	—		20	10-4	20	2-5
Riboflavine-5′-phosphate	20	0-2	20	0-0	3	0-0	20	13-4	20	3-4
Adenosine-5′-sulphate	25	6-1	25	3-1	25	0-0	25	10-5	20	3-4
Amino acids (d)	15	0-0	—		—		15	11-1	—	
$NaHCO_3$ (buffer)	65	0-0	—		—					
KH_2PO_4-Na_2HPO_4 (buffer)	20	0-0	—		—					

* Numbers satiate are shown on the left side of hyphens and those partially fed are on the right in the columns (f).

† These are dissolved in 0·15 M NaCl (pH *ca.* 7) and fed forcibly.

(a), (b) Containing 1·0 per cent and 6·1 per cent of adenosine monophosphate, respectively.

(c) Containing 3·5 per cent and 10·7 per cent respectively of adenosine mono- and di-phosphate.

(d) The data are commonly applicable to each of the following six amino acids: glycine, glutamic acid, cysteine, arginine, tyrosine, and histidine.

At the concentration of 1×10^{-4} M, 5′-adenylic acid brought about engorgement in 49 per cent of the insects exposed and partial feeding in 15 per cent (Table 6). The latter may be considered to be mostly, if not exclusively, caused by weaker stimulation by the adenylic acid, since the ratio of partial feeding to full feeding increases as the concentration approaches the threshold value of engorgement, which seems to exist in the neighbourhood of 10^{-5} M. Under the present conditions of feeding, stimulus by the adenylic acid is at its full strength at 1×10^{-4} M and seems to remain nearly constant up to 1×10^{-2} M. Why the stimulative power is not enhanced with the increasing concentration of the acid will be discussed later.

In an examination not included in Table 6, a non-buffered 10^{-2} M 5′-adenylic acid was found to be evidently far less acceptable than a 10^{-3} M one, most probably owing to the higher acidity of the former solution. In the light of the previous work by FRINGS (1946) and CHADWICK and DETHIER (1947), the stimulative power of

acids in insect chemoreception is dependent not solely upon the hydrogen-ion concentration, but also upon the property and concentration of the anions. It is not unwarranted, therefore, to suppose that an excess rise of the 5′-adenylic acid concentration tends to reduce acceptability of the compound until this becomes rejected by the mosquito regardless of the solution being neutralized. Such a trend is nothing but what is observed with NaCl (Sections A, 1; B, 2), besides being reported by BUTLER (1940) and FRINGS (1946). Thus considered, the type of stimulation caused by 5′-adenylic acid is no longer unique in the physiology of contact chemoreception of insects as it first appears. It may belong to the same type which characterizes some of the acids and salts possessed of the double modality (DETHIER and CHADWICK, 1948), only differing from them in that a peculiarity as it becomes exceedingly acceptable to the mosquito over a wide range of very low concentrations.

It would seem somewhat curious that in the present experiments 0·15 M NaCl proved entirely unacceptable (Table 7). However, the solutions used here had been buffered with either 0·001 M $NaHCO_3$ or 0·001 M phosphates. In view of the trivial effectiveness of NaCl, there might be a good possibility that the buffering compounds had cancelled out the acceptability of the solution. By analogy, some of the negative results obtained with several acids might possibly be derived from the use of buffered solutions. Apparently all the six amino acids were not accepted in accordance with the statements by VON FRISCH (1934). It cannot be concluded, however, that several cases of aberrant positive responses recorded in Table 7 are always caused by a contamination of 5′-adenylic acid. Since a similar instance has also occurred in the tests of fractionated blood extracts (Fig. 5), the above results may rather be the true indication of slight acceptability of the test compounds.

Besides 5′-adenylic acid there are four compounds which exert marked stimulation on the mosquito; i.e. 5′-deoxyadenylic acid, adenosine di- and tri-phosphate, and adenosine-5′-sulphate (Table 7). On account of the contamination of 5′-adenylic acid in solutions of adenosine diphosphate, the net influence of the latter is not accurately measurable, though its effectiveness is undoubted. The ambiguity increases in considering the effect of the triphosphate ally, but most probably this compound may also possess a certain degree of stimulatory effect. The 1×10^{-4} M solution of the triphosphate produced a better result than the 1×10^{-5} M diphosphate and so did the 1×10^{-5} M triphosphate as compared with 1×10^{-6} M diphosphate, on which 1 engorged and 3 partially fed out of 20 exposed. This fact seems somewhat difficult to explain exclusively by the 3·5 per cent and 10·7 per cent contaminations respectively of the mono- and di-phosphate in the triphosphate solutions.

Table 7 also indicates a few interesting correlations between acceptability of the compounds and their structures. The nucleotide-like constitution involving the adenine moiety is of fundamental importance to the rise of stimulative power. Either the conversion of the ribose group to deoxyribose or the replacement of the phosphate group by sulphate reduces the acceptability; but the reduction is

not serious. This is in marked contrast to the consequence of a migration of the phosphate group from the 5′ position to the 2′ or 3′, which reduces almost entirely the action of adenine nucleotide. This idea is also supported by the previous finding that the hydrolysates of yeast pentose nucleic acid are virtually ineffective.

The physiological significance. It may be inquired to what extent the foregoing results resemble the blood stimulation under natural circumstances. The feeding activity on adenosine-5′-phosphates is sufficiently comparable with that on sugar-free intact erythrocytes (Tables 6 and 7 vs. Fig. 1(a), $F-$). The percentages that satiate, though not extremely high, seem reasonable for conditions of the forcible feeding. Unfortunately, data for the adenine nucleotide content of ox erythrocytes are not available. In freshly shed human blood, total adenine nucleotides per litre erythrocytes are reported to amount to about $1{\cdot}5 \times 10^{-3}$ mole. They exist mostly in the form of adenosine triphosphate (OVERGAARD-HANSEN *et al.*, 1957). The amount is about four times as large as that calculated for the ox blood extract obtained from the same volume of the erythrocytes. However, since the extraction was not originally intended for the measurement of the nucleotide content, the smaller amount in the latter would not be surprising. Granted that nearly equal amounts of adenine nucleotides are present in ox erythrocytes as in human ones, and assuming that a 50-fold dilution of the cells represents the threshold of gorging, the nucleotides have to be effective down to the concentration of $0{\cdot}3 \times 10^{-4}$ M. This value seems quite reasonable provided that all the adenine nucleotides contained in the erythrocytes are available for feeding stimulation as efficiently as 5′-adenylic acid given in the form of a solution. According to CHEN and JØRGENSEN (1956), adenosine triphosphate in shed human blood breaks down rapidly into hypoxanthine and the degradation is accelerated by haemolysis. It seems likely that the 'ageing phenomenon' observed in the stimulatory effect of haemolysed ox erythrocytes (Section A, 3) has a close relation to the above reaction.

A question arises as to how the mosquito can make use of the blood adenine nucleotides so readily. As for the experimental result, ambiguity remains in the effectiveness of adenosine triphosphate. Even if the compound were equally as stimulative as the mono- or di-phosphate, it is hardly conceivable that the compound diffuses out of the blood cells so freely into the sense organs of the insect as when it is present in saline. Since repeated washings of erythrocytes do not reduce their acceptability, naturally occurring adenine nucleotides should be bound to the cells with considerable firmness. It follows that there must be some means for the mosquito to release the compounds from the cells during the period of aspiration of a blood meal. Whether the saliva or the surface of the sense organs does this awaits further investigation. In this respect the particulate nature of erythrocytes might also be related to the acceptability of the cells, not because of the possibility of tactile stimulation, but because of an action such as the presence of stroma providing the most suitable condition for erythrocytes to retain their adenine nucleotides and to liberate them promptly during the passage to the midgut of the mosquito.

Acknowledgements—This work was performed with the aid of valuable suggestions as well as a supply of chemicals by Professors ATSUSHI TAKAMIYA, JURO ISHIDA, and TAKASHI FUJII of Tokyo University, Professor FUJIO EGAMI of Nagoya University, Dr. MASASHI NAGAHISA and Dr. TETSUO SATO of the Tokyo Institute of Technology, to all of whom the writer acknowledges his great indebtedness. His special thanks are due to Dr. SOICHI FUKUDA of the Katakura Sericultural Experiment Station for his constant encouragement and to Mr. KOICHI OGURA for his timely advice and assistance. The writer also wishes to express his gratitude to Dr. HELEN L. T. DURKEE of the American Mosquito Control Association through whose courtesy some of the important literature became accessible, and to Professor V. G. DETHIER who kindly examined this manuscript.

REFERENCES

ANSON M. L. and MIRSKY A. E. (1930) Protein coagulation and its reversal. The preparation of insoluble globin, soluble globin and heme. *J. gen. Physiol.* **13**, 469–476.

BISHOP A. and GILCHRIST B. M. (1946) Experiments upon the feeding of *Aëdes aegypti* through animal membranes with a view to applying this method to the chemotherapy of malaria. *Parasitology* **37**, 85–100.

BUTLER C. G. (1940) The choice of drinking water by the honeybee. *J. exp. Biol.* **17**, 253–261.

CHADWICK L. E. and DETHIER V. G. (1947) The relationship between chemical structure and the response of blowflies to tarsal stimulation. *J. gen. Physiol.* **30**, 255–262.

CHARGAFF E., MAGASANIK B., VISCHER E., GREEN C., DONIGER R., and ELSON D. (1950) Nucleotide composition of pentose nucleic acids from yeast and mammalian tissues. *J. biol. Chem.* **186**, 51–67.

CHEN P. S. and JØRGENSEN S. (1956) Formation of hypoxanthine from adenosine triphosphate in shed human blood. *Acta pharm. tox. Kbh.* **12**, 369–378.

COHN W. E. (1950) The anion-exchange separation of ribonucleotides. *J. Amer. chem. Soc.* **72**, 1471–1478.

DAY M. F. (1954) The mechanism of food distribution to midgut or diverticula in the mosquito. *Aust. J. biol. Sci.* **7**, 515–524.

DETHIER V. G. and CHADWICK L. E. (1947) Rejection thresholds of the blowfly for a series of aliphatic alcohols. *J. gen. Physiol.* **30**, 247–253.

DETHIER V. G. and CHADWICK L. E. (1948) Chemoreception in insects. *Physiol. Rev.* **28**, 220–254.

EGAMI F. and TAKAHASHI N. (1955) Syntheses of adenosinesulfuric acids. *Bull. chem. Soc. Jap.* **28**, 666–668.

FISK F. W. (1950) Studies on proteolytic digestion in adult *Aedes aegypti* mosquitoes. *Ann. ent. Soc. Amer.* **43**, 555–572.

FRINGS H. (1946) Gustatory thresholds for sucrose and electrolytes for the cockroach, *Periplaneta americana* (Linn.). *J. exp. Zool.* **102**, 23–50.

FRINGS H. and HAMRUM C. H. (1950) The contact chemoreceptors of adult yellow fever mosquitoes, *Aedes aegypti*. *J. N.Y. ent. Soc.* **58**, 133–142.

VON FRISCH K. (1934) Über den Geschmackssinn der Biene. Ein Beitrag zur vergleichenden Physiologie. *Z. vergl. Physiol.* **21**, 1–156.

FÜLLEBORN F. (1908) Über Versuche an Hundefilarien und deren Übertragung durch Mücken. *Arch. Schiffs- u. Tropenhyg.* **12**, Beih. **8**, 313–351.

FÜLLEBORN F. (1932) Über den Saugakt der Stechmücken. *Arch. Schiffs- u. Tropenhyg.* **36**, 169–181.

HASLINGER F. (1935) Über den Geschmacksinn von *Calliphora erythrocephala* Meigen und über die Verwertung von Zuckern und Zuckeralkoholen durch diese Fliege. *Z. vergl. Physiol.* **22**, 614–640.

VOL. 3 1959

HASSETT C. C., DETHIER V. G., and GANS J. (1950) A comparison of nutritive value and taste thresholds of carbohydrates for the blowfly. *Biol. Bull., Woods Hole* **99**, 446–453.

HOSOI T. (1954a) Mechanism enabling the mosquito to ingest blood into the stomach and sugary fluids into the oesophageal diverticula. *Annot. zool. jap.* **27**, 82–90.

HOSOI T. (1954b) Egg production in *Culex pipiens pallens* Coquillett. I–V. *Jap. J. med. Sci. Biol.* **7**, 57–73, 75–81, 111–127, 129–134, 231–240.

KADLETZ N. A. and KUSMINA L. A. (1929) Experimentelle Studien über den Saugprozess bei *Anopheles* mittels einer zwangsweisen Methode. *Arch. Schiffs- u. Tropenhyg.* **33**, 335–350.

KIKUCHI G. (1953) Hemoglobin. *In* EGAMI *et al.*, *Hyojun-Seikagaku-Jikken* p. 112–124, Bunkodo, Tokyo.

KISTLER P., BURI A., and NITSCHMANN H. (1953) Über Gewinnung und Eigenschaften von humanem Globin. *Helv. chim. acta* **36**, 1058–1070.

KRIJGSMAN B. J. (1930) Reizphysiologische Untersuchungen an blutsaugenden Arthropoden im Zusammenhang mit ihrer Nahrungswahl. I Teil. *Stomoxys calcitrans. Z. vergl. Physiol.* **11**, 702–729.

KRIJGSMAN B. J. and WINDRED G. L. (1930) Reizphysiologische Untersuchungen an blutsaugenden Arthropoden im Zusammenhang mit ihrer Nahrungswahl. II Teil. *Lyperosia exigua. Z. vergl. Physiol.* **13**, 61–73.

LETSCHE E. (1924) Aufarbeitung des Blutes zur Gewinnung und Bestimmung seiner organischen Einzelbestandteile. *In* ABDERHALDEN, *Handbuch der biologischen Arbeitsmethoden* **4** (3) 589–684, Urban and Schwarzenberg, Berlin and Wien.

MACGREGOR M. E. and LEE C. U. (1929) Preliminary note on the artificial feeding of mosquitoes. *Trans. R. Soc. trop. Med. Hyg.* **23**, 203–204.

MACGREGOR M. E. (1930) The artificial feeding of mosquitoes by a new method which demonstrates certain functions of the diverticula. *Trans. R. Soc. trop. Med. Hyg.* **23**, 329–331.

MACGREGOR M. E. (1931) The nutrition of adult mosquitoes: preliminary contribution. *Trans. R. Soc. trop. Med. Hyg.* **24**, 465–472.

MINNICH D. E. (1921) An experimental study of the tarsal chemoreceptors of two nymphalid butterflies. *J. exp. Zool.* **33**, 173–203.

MINNICH D. E. (1929) The chemical sensitivity of the legs of the blow-fly, *Calliphora vomitoria* Linn., to various sugars. *Z. vergl. Physiol.* **11**, 1–55.

MINNICH D. E. (1931) The sensitivity of the oral lobes of the proboscis of the blowfly, *Calliphora vomitoria* L. to various sugars. *J. exp. Zool.* **60**, 121–139.,

MORITA H., DOIRA S., TAKEDA K., and KUWABARA M. (1957) Electrical response of contact chemoreceptor on tarsus of the butterfly, *Vanessa indica*. *Mem. Fac. Sci. Kyūsyū Univ. (E.)* **2**, 119–139.

OVERGAARD-HANSEN K., JØRGENSEN S., and PRAETORIUS E. (1957) Rephosphorylation produced by inosine and adenosine of adenosine monophosphate and adenosine diphosphate in human erythrocytes. *Nature, Lond.* **179**, 152–153.

ROY D. N. and GHOSH S. M. (1940) On the results of forcible feeding of mosquitoes. *J. Malar. Inst. India* **3**, 253–262.

SCHMIDT A. (1938) Geschmacksphysiologische Untersuchungen an Ameisen. *Z. vergl. Physiol.* **25**, 351–378.

TREMBLEY H. L. (1952) The distribution of certain liquids in the esophageal diverticula and stomach of mosquitoes. *Amer. J. trop. Med. Hyg.* **1**, 693–710.

WEIS L. (1930) Versuche über die Geschmacksrezeption durch die Tarsen des Admirals, *Pyrameis atalanta* L. *Z. vergl. Physiol.* **12**, 206–248.

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 219 *to* 229. *Pergamon Press Ltd., London. Printed in Great Britain*

STUDIES ON THE MECHANISM OF TYROSINASE ACTIVATION IN THE HOUSEFLY, *MUSCA VICINA* MACQ.

EIJI OHNISHI*

Department of Biology, Faculty of Science, Tokyo Metropolitan University, Setagayaku, Tokyo, Japan

(*Received* 4 *September* 1958)

Abstract—It has been reported that tyrosinase of *Drosophila* or *Musca* occurs as a proenzyme, which becomes active by the action of a protein activator, and that the level of tyrosinase activity finally attained was considerably lower when the activation was allowed to take place at 25°C, instead of 0°C. Detailed studies on this temperature effect, described in the present paper, revealed the presence of an inactivation process of the proenzyme besides the activation process. From the results of kinetic experiments, a close relationship between the production of the activator and that of tyrosinase is suggested.

VOL. 3 1959

INTRODUCTION

IT has been reported (BODINE *et al.*, 1938, 1944) that tyrosinase in the diapausing egg of the grasshopper, *Melanoplus differentialis*, occurs as an inert proenzyme, which can be activated by treating it with a variety of surface active substances, or heavy metals, or high temperature.

Recently, another type of tyrosinase activation was found in insects: tyrosinase of *Drosophila* occurs as a proenzyme, which becomes active by the action of a protein activator (OHNISHI, 1953, 1954; HOROWITZ and FLING, 1955). The same type of tyrosinase activation was also found in the pupae of the housefly, *Musca vicina* Macq. and some properties of the activation process were presented in a previous paper (OHNISHI, 1957).

The present paper deals with studies on the same material in attempts to clarify the effect of temperature on the activation process. Making use of the temperature effect, some kinetic experiments were also conducted.

MATERIALS AND METHODS

For the source of the enzyme system, acetone powder as well as fresh material was used. A culture method for the housefly was described previously (OHNISHI, 1957). Acetone powder was made by homogenizing pupae in roughly 10 parts of ice-cold acetone and washing with absolute acetone. Drying was achieved in a stream of dried air by the device described by TAMIYA (1955).

* Present address: Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California, U.S.A.

Extraction for the activation experiments was made with ice-cold M/30 phosphate buffer (pH 7·0) by homogenizing either in a blender (in the case of fresh pupae) or in a glass mortar (in the case of acetone powder) and centrifuging the homogenate at 3000 rev/min for 3 min. The activation process was followed by sampling at intervals from the extract, which was kept at the desired temperature. Usually, the time at which homogenizing was done was taken as the zero time for the activation process. But in the experiments where the activation proceeds rapidly at the beginning, the homogenate was at first made with cold M/30 Na_2HPO_4, which suppresses activation during the extraction procedure, and the homogenate was neutralized with an appropriate amount of cold M/30 KH_2PO_4 at the zero time for the activation process.

Tyrosinase activity was determined by the colorimetric method of HOROWITZ and SHEN (1952). Tyrosinase was allowed to react with dopa at pH 6·0 for 5 min at 30°C, and was blocked by the addition of 2 drops of N/5 KCN. The intensity of the colour of dopachrome, produced by the action of tyrosinase, was measured by a Leitz Photometer with 445 mμ filter.

EXPERIMENTAL RESULTS

(I) *Effect of temperature on tyrosinase activation*

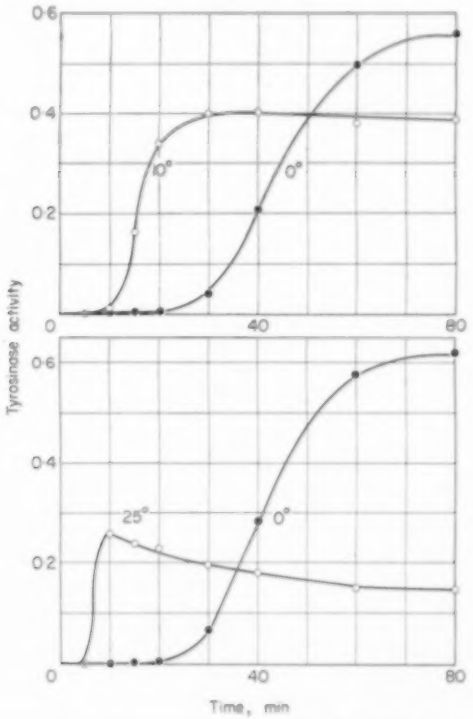


FIG. 1. Effect of temperature on tyrosinase activation. Extracts made from fresh pupae were divided into two parts and each part was exposed to the temperature indicated.

It was noticed by HOROWITZ and FLING that the level of tyrosinase activity finally attained was considerably lower when the activation was allowed to take place at 25°C instead of 0°C. They suggested that the effect might be due to the destruction of the activating system at 25°C. In order to analyse the temperature effect, the following experiments were conducted.

In the first series of experiments, the activation was allowed to take place at various temperatures ranging from 0°C to 40°C. Each extract, made from fresh pupae, was divided into two parts and the one was incubated at the varied temperature and the other was kept at 0°C for comparison. The course of the activation process was followed by sampling and measuring the activities at intervals. Two examples are shown in Fig. 1. It is clear from the figure that at the high temperatures there is a marked shortening of the lag period and a reduction of the final activity.

The relation between the temperature and the final yield of tyrosinase is shown in Fig. 2, in which the final tyrosinase activity at the given temperature is expressed as the percentage of that produced at 0°C. A remarkable fact is that the final yield of tyrosinase was affected even at a relatively low temperature such as 5°C.

VOL. 3 1959

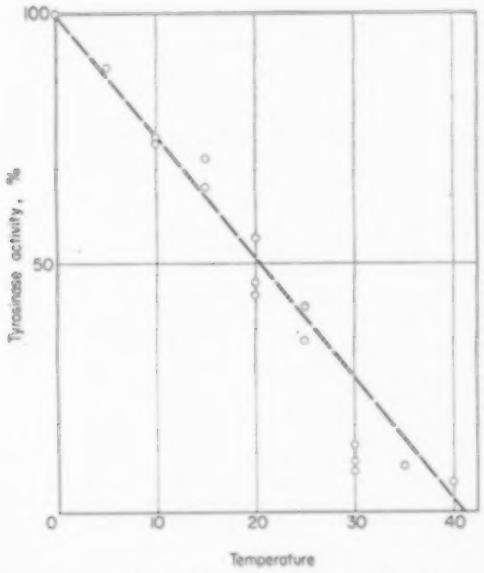


FIG. 2. Effect of temperature on the final yield of tyrosinase. Tyrosinase activity at the given temperature is expressed as the per cent activity of that obtained in the activation at 0°C.

For the analysis of the temperature effect, it is necessary to know which of the reactants, viz. the proenzyme or the activator, is limiting the final level of tyrosinase activity.

When the activation process was allowed to proceed at 0°C, the limiting factor was determined by the addition of 5 mg of activator preparation.*

As shown in Fig. 3, the activator preparation caused a marked shortening of the lag period, but the final yield of tyrosinase activity was not affected by the addition of the activator. This fact seems to indicate that the concentration of the proenzyme is limiting the final level of tyrosinase activity when the activation is allowed to take place at 0°C.

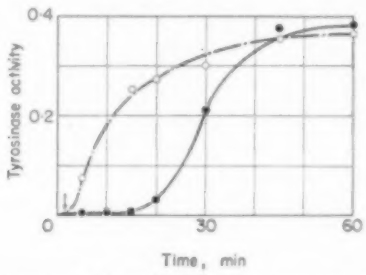


FIG. 3. Tyrosinase activation with and without activator preparation. The extract was divided into two parts; one of them was mixed with the activator preparation (broken line) and the other was mixed with distilled water (solid line) at the time indicated by the arrow.

To determine whether the proenzyme or the activator is limiting the final yield of tyrosinase at high temperature, two types of experiments were conducted.

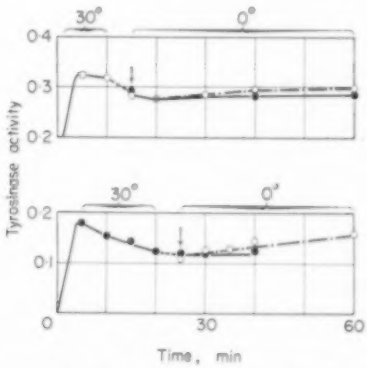


FIG. 4. Thermal inactivation of the proenzyme at 30°C. Fresh extract was incubated at 30°C for 10–20 min and then cooled to 0°C. Half of it was mixed with the activator preparation (broken line) and the other half was mixed with distilled water (solid line) at the time indicated by the arrow.

* The activator preparation was made as follows: Acetone powder was extracted with phosphate buffer (pH 8·0), containing HCN of 1/10 normality in a final concentration and saturated ammonium sulphate solution was added to precipitate the activator (60 per cent was sufficient). The precipitate was dissolved in the phosphate buffer containing N/10 HCN and the solution was dialysed against tap water. The dialysed solution was centrifuged, and the supernatant, which was usually devoid of tyrosinase activity, was used as the activator preparation.

Firstly, the activation was allowed to take place at 30°C for 10–20 min and then cooled to 0°C. Half of the extract was mixed with the activator preparation (5 mg), and the other half was mixed with distilled water for comparison.

If the activator is denatured and the proenzyme is left unaffected at the high temperature, the activation of the extract should resume at the time when the activator is provided at low temperature. On the other hand, the resumption of the activation cannot be expected if the proenzyme is affected at high temperature.

The results of the experiments shown in Fig. 4 support the second alternative, i.e. that the proenzyme is affected at high temperature.

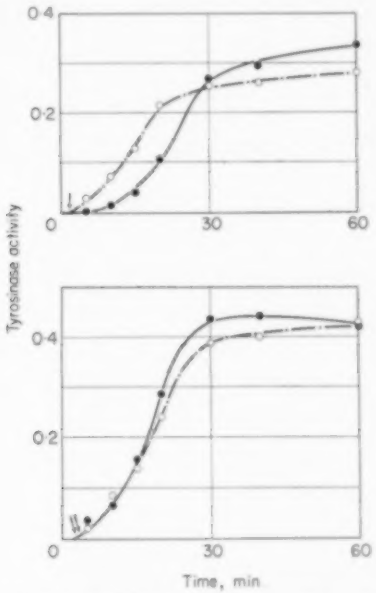


FIG. 5. Effect of heat treatment (30°C for 30 min) on the activator preparation. Upper figure indicates the activation of the extract with (broken line) and without (solid line) heat-treated activator preparation. Lower figure indicates the activation of the extract, with heat-treated (broken line) and non-treated (solid line) activator preparation.

Secondly, thermal stability of the activator preparation was examined by incubating it at 30°C for 30 min before the addition to the system. The incubated preparation still possessed the ability to shorten the lag period of the fresh extract, which was allowed to become active at 0°C (Fig. 5, upper figure). Another experiment of a similar type was conducted to secure further information regarding the thermal stability of the activator. The extract prepared from fresh pupae was divided into two parts and these were mixed with heat-treated (30°C for 30 min) and non-treated activator preparation. As seen in Fig. 5 (lower figure), both extracts behaved similarly, indicating that heat treatment of the activator preparation did not affect significantly the activating capacity.

From these experiments, it may be concluded that the effect of high temperature, acting to reduce the final yield of tyrosinase activity, is the result of destruction or inactivation of the proenzyme.

TABLE 1—THERMOSTABILITY OF THE PROENZYME UNDER CONDITIONS IN WHICH THE ACTIVATION IS SUPPRESSED

Heat treatment		Tyrosinase activity
Temperature (°C)	Time (min)	(per cent*)
20	30	103
25	30	100
30	30	58

* Per cent activity of that possessed before the heat treatment. Tyrosinase activity was measured after dialysing out the salt and the buffer.

To sum up, the bulk of the proenzyme is activated to the active tyrosinase where the activation is allowed to take place at 0°C, whereas most of the proenzyme is converted to an inactive product at high temperature. This fact can be interpreted in terms of temperature coefficient, i.e. the temperature coefficient of the inactivation process is greater than that of the activation process, thus causing the former process to prevail at high temperature.

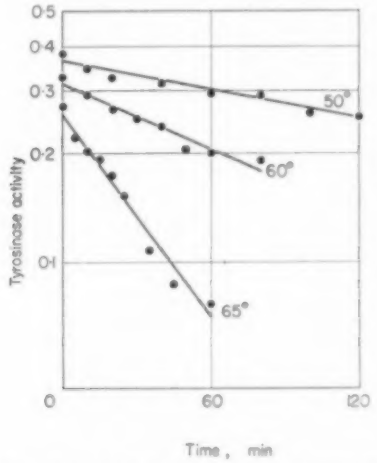


FIG. 6. Thermal stability of the activated enzyme (tyrosinase). Tyrosinase activity is plotted on a logarithmic scale.

The next step in the analysis of the nature of the temperature effect is to know what the inactivation process is. One of the possibilities is the thermal denaturation

of the proenzyme molecule. Unfortunately, it is as yet not possible to estimate directly the thermal stability of the proenzyme at neutral pH, because of the difficulty in obtaining an activator-free proenzyme preparation (activation occurs in this condition). Thermal stability is, therefore, inferred (a) from the thermal stability of the proenzyme in the medium of high alkalinity (pH 10·0) and high ionic strength ($\mu = 1{\cdot}0$) (in this condition the activation process is suppressed completely) and (b) from the thermal stability of the activated tyrosinase.

The thermal stability of the proenzyme in the above-mentioned condition is shown in Table 1, which indicates that the proenzyme is not so labile as to be affected at relatively low temperature. Although it was affected by treating at 30°C for 30 min, this fact does not necessarily mean that the proenzyme is relatively labile at high temperature, since the alkalinity at which the stability was estimated is not favourable to most proteins.

Thermal stability of the activated enzyme is illustrated in Fig. 6, which indicates the relatively high stability of the enzyme.

Judging from these results, the inactivation of the proenzyme at high temperature may not be the thermal denaturation of the proenzyme.

VOL. 3 1959

(II) *Increase in the activator concentration during the activation process and close relation of the increase to tyrosinase production*

If the hypothesis of HOROWITZ and FLING is accepted, the following points can be expected from the autocatalytic reaction formula (proenzyme + activator → tyrosinase + 2 activator).

Increase in tyrosinase activity during the activation process will proceed in a sigmoid fashion (provided that the initial concentration of the activator is less than that of the proenzyme). The inflexion point of the curve is the point at which the two reactants, viz. the activator and the proenzyme, have the same concentration. Therefore, if the concentration of the activator is increased experimentally above that of the proenzyme at the onset of the reaction, the increase in the tyrosinase activity will no more follow the sigmoid curve, but will fit a convex curve, which has no inflexion point (upper part of the sigmoid curve). After the completion of the activation, the concentration of the activator will exceed that of tyrosinase, activated during the reaction, by the initial concentration of the activator.

To test these kinetic predictions, the following experiments were made. In order to ensure the constancy of the results, acetone powder was used as the source of the enzyme system. All the reactions were allowed to take place at 0°C to avoid temperature effects.

In the first experiments, 60 mg of acetone powder was extracted with 5 ml of ice-cold M/30 phosphate buffer (pH 7·0), and the extract was allowed to become active. One such experiment is shown in Fig. 7 (solid line), as a typical sigmoid curve. The initial concentration of the activator, which was estimated from the inflexion points of seven experiments, is in the neighbourhood of 15–25 per cent of that of the proenzyme. Therefore, the final concentration of the activator may be 115–125 per cent of that of tyrosinase.

In the second experiment, the first extract, made from 60 mg of acetone powder and allowed to complete the activation, was mixed with the second proenzyme-activator system (made from the same amount of acetone powder) at the onset of the second activation process. From the reasoning presented above, the increase in tyrosinase activity is expected to follow the convex curve without an inflexion point, instead of a sigmoid curve. Experimental results confirmed the expectation, as shown in Fig. 7 (broken line). In the figure, the tyrosinase activity of the extract, which had completed its activation before the addition of the reaction system, is subtracted.

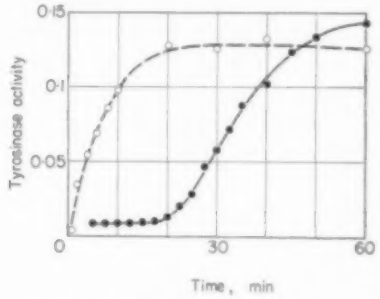


FIG. 7. Time-activity curve of the extract, made from acetone powder. For the explanation of the curves, see text. Tyrosinase activity of the extract, which had completed its activation before the addition to the reaction system, is subtracted in the figure.

According to the autocatalytic reaction formula of HOROWITZ and FLING, the activator is contained in and generated from the proenzyme molecule, along with the active tyrosinase, in the course of the activation process. If so, it may be possible that the production of the activator is also suppressed at high temperature at the same time as the production of the active tyrosinase.

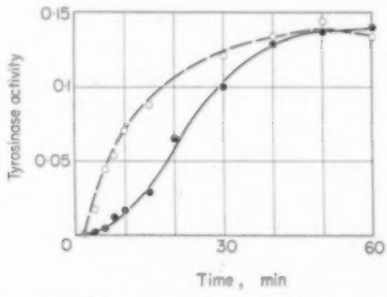


FIG. 8. Time-activity curve of the extract, made from acetone powder. For the explanation of the curves, see text. Tyrosinase activity of the extract, which had completed its activation before the addition to the reaction system, is subtracted in the figure.

In order to ascertain this point, the third experiment was conducted. The design of the experiment was the same as that of the second experiment, except

that the first activation process, which is to supply the activator to the second reaction system, was allowed to take place at 30°C. If the above supposition is correct, it may again be expected to have a sigmoid curve. Experimental results fitted well the expectation, as shown in Fig. 8 (solid line). In the figure, the tyrosinase activity of the extract, which had completed its activation before the addition of the reaction system, is subtracted.

To make sure that the effect of high temperature is not due to the thermal destruction of the already formed activator, the fourth experiment was made. The design of this experiment was the same as that of the second one, except that the first extract, which was allowed to complete the activation at 0°C, was incubated at 30°C for 30 min before the addition to the second system. As shown in Fig. 8 (broken line), the same activation curve (the convex one) as that in the second experiment was obtained. This fact indicates that the effect of high temperature is to suppress the production of the activator, but not to inactivate the already produced activator.

VOL. 3 1959

DISCUSSION

From the detailed studies concerning the temperature effect on the activation process, it becomes necessary to introduce a third component, which inactivates the proenzyme, to the tyrosinase system of Diptera.

In a previous paper (OHNISHI, 1957), it was shown that the activator preparation cannot only shorten the lag period, but also cause an increase in the final yield of tyrosinase. This fact can be explained by the competition for the proenzyme between the activation- and inactivation-processes at high temperature employed (30°C).

The nature of the inactivation is not known, but the possibility of thermal denaturation of the proenzyme molecule is not likely from the following evidence. Firstly, judging from the thermal stability of the activated enzyme and that of the proenzyme under conditions that suppress the activation, the proenzyme is not likely to be labile at relatively low temperature, such as 5–10°C. Secondly, it is also unusual for proteins to be denatured at such low temperatures in a short time, and, further, the temperature coefficient of protein denaturation is generally large, so that denaturation becomes evident suddenly within a very narrow temperature range. This fact cannot be reconciled with the inactivation process, which occurs over a wide range of temperature.

It was previously suggested by the author that activation of a similar kind also occurs in the tyrosinase system of silkworm body fluid (1954). For comparison, the activation of this material was allowed to take place at 30°C as well as 0°C. As shown in Fig. 9, there is a shortening of the lag period in the activation at 30°C, but the suppression of the final yield of tyrosinase at high temperature was not encountered in this material. The fact seems to suggest that in the tyrosinase system of the silkworm, inactivation of the proenzyme does not exist.

For the mechanism of the activation, HOROWITZ and FLING proposed a modified autocatalytic reaction scheme, in which active tyrosinase is a product of

the reaction and has no role in the process. The experiments presented in this paper have confirmed the increase in the concentration of the activator during the activation process. It is further shown that the increase of the activator is suppressed at high temperature, where the production of the active enzyme is also

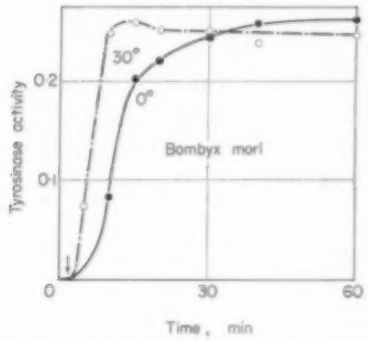


FIG. 9. Effect of temperature on the activation of tyrosinase in the body fluid of the silkworm.

suppressed. The fact suggests a close relation between the production of tyrosinase and that of the activator. It thus supports the autocatalytic hypothesis of tyrosinase activation.

Most of the enzymes which are found to occur as proenzymes are extracellular enzymes; for example, proteolytic enzymes of the mammalian digestive tract. These are stored as the proenzymes in the cells. The biological meaning of the occurrence of protyrosinase in the body fluid in insects may be assumed to be either (a) that tyrosinase is produced in the body fluid as the proenzyme but exerts its activity at other loci, such as in hypodermis or in cuticle, or (b) that tyrosinase works only at certain stages in development, for instance at pupation, and is stored as the proenzyme during the other stages.

SUMMARY

The effect of temperature on the final yield of tyrosinase was studied. When tyrosinase activation was allowed to take place at various temperatures ranging from 0°C to 40°C, the yield of tyrosinase was reduced with increase of temperature. The effect is due to the inactivation of the proenzyme, although the inactivation is not likely to be the thermal denaturation of the enzyme. Increase in the activator concentration was observed during the activation process. The increase in the activator concentration was suppressed at 30°C, along with that of tyrosinase, suggesting a close relationship between the production of the activator and that of tyrosinase.

Acknowledgements—The author wishes to express his gratitude to Professors Y. AKITA and T. FUJII for their suggestions and encouragement. Thanks are also due to Professor

N. H. HOROWITZ of California Institute of Technology for valuable criticism and for reading the manuscript.

REFERENCES

BODINE J. H. and ALLEN T. H. (1938) Enzymes in ontogenesis (Orthoptera)—V. Further studies on the activation of the enzyme, tyrosinase. *J. cell comp. Physiol.* **12**, 71–84.

BODINE J. H., TAHMITIAN T. N., and HILL D. L. (1944) Effect of heat on protyrosinase. Heat activation, inhibition and injury of protyrosinase and tyrosinase. *Arch. Biochem.* **4**, 403–12.

HOROWITZ N. H. and FLING M. (1955) The autocatalytic production of tyrosinase in extracts of *Drosophila melanogaster*. *Amino acid metabolism*. Edited by W. D. MCELROY and B. GLASS, Johns Hopkins Press, Baltimore, 207–18.

HOROWITZ N. H. and SHEN S-C. (1952) Neurospora tyrosinase. *J. biol. Chem.* **197**, 513–20.

OHNISHI E. (1953) Tyrosinase activity during puparium formation in *Drosophila melanogaster*. *Jap. J. Zool.* **11**, 69–74.

OHNISHI E. (1954) Activation of tyrosinase in *Drosophila virilis*. *Annot. zool. jap.* **27**, 88–93.

OHNISHI E. (1958) Tyrosinase activation in the housefly, *Musca vicina* M. *Jap. J. Zool.* **12**, 179–88.

TAMIYA N. (1955) *Koso-kenkyuho*. Edited by S. Akabori, Asakura Book Co., Tokyo.

VOL.
3
1959

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 230 *to* 242. *Pergamon Press Ltd., London. Printed in Great Britain*

ELECTRICAL PROPERTIES OF THE COCKROACH GIANT AXON

TERUO YAMASAKI and TOSHIO NARAHASHI

Laboratory of Applied Entomology, Faculty of Agriculture, University of Tokyo, Tokyo, Japan

(*Received* 6 *October* 1958)

Abstract—The electrical properties of the cockroach giant axons have been examined by means of intracellular microelectrodes. Membrane resistance and membrane capacity were estimated by recording electrotonic potentials produced by square pulses of polarizing current. The membrane capacity was found to be relatively high as compared with other kinds of nerve fibres. A delayed rectification was observed with a strong cathodal current. The maximum rate of rise of the action potential was decreased by depolarization, whereas it underwent little change by hyperpolarization. The changes in after-positivity and after-negativity under electrotonus suggest that the mechanisms of their production are different. The inward sodium current during an impulse was calculated on the basis of the sodium theory. The peculiarities of the electrical properties of the roach giant axon are discussed.

WHEREAS the electrical properties of nerve and muscle fibres have received much attention, only a few data are available for those of insect nerves. Resting and action potentials of the giant axons from the cockroach have been recorded by means of intracellular microelectrodes (BOISTEL and CORABOEUF, 1954; CORABOEUF and BOISTEL, 1955; YAMASAKI and NARAHASHI, 1957, 1959). It was found that there is no substantial difference between the roach axon and other nerve fibres when the external sodium and potassium concentrations are varied (YAMASAKI and NARAHASHI, 1959). In order to elucidate the mechanism involved in the excitation of the cockroach giant axon, its electrical properties must be investigated. The present paper gives the results of experiments concerning the electrical properties of the cockroach giant axons as examined by intracellular microelectrodes.

METHODS

Insects and nerve preparations are the same as those used in our previous works (YAMASAKI and NARAHASHI, 1957, 1959). In order to improve high frequency responses a compensation circuit as described before was also employed (YAMASAKI and NARAHASHI, 1959).

Stimulation and recording

One and the same microelectrode inserted in the axon was used for both stimulating and recording. In order to eliminate the potential drop across the resistances other than nerve

membrane, the Wheatstone bridge circuit used by ARAKI and OTANI (1955) and FRANK and FUORTES (1956) was employed (Fig. 1). A microelectrode was inserted in an axon while the input of 'voltage' measuring amplifier was being connected with *B*. This connexion made the bridge circuit inoperative. When it was confirmed that the impaled axon was capable of generating action potentials of normal amplitude for a period of more than 10 min after impalement, the input was then connected with *C*, thus making the bridge circuit operative. Immediately after this, compensation voltage was adjusted by R_5 so as to cancel the resting potential. Calibration potential was applied through the resistance of 500 Ω, R_4. Bridge

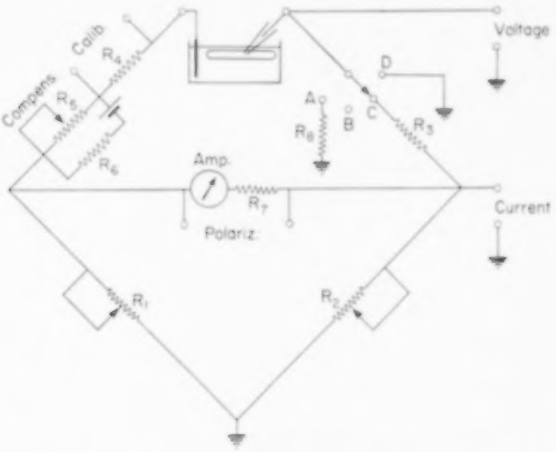


FIG. 1. Diagram of arrangement used for polarizing and recording. R_1 1 KΩ, R_3 10 KΩ, R_2 85 MΩ, R_4 500 Ω, R_5 100 Ω, R_6 300 Ω, R_7 104 KΩ, and R_8 10·37 MΩ.

balancing was performed by adjusting the two variable resistances, R_1 and R_2, in the lower limbs of the bridge so as to eliminate the potential drop across resistances other than nerve membrane. This could easily be performed because the electrotonic potentials arising from the membrane showed a slow rise and fall whereas the potentials from other resistances showed a very sharp rise and fall. If necessary, applied polarizing voltages were measured by knowing the current intensities passing through R_7. Applied currents were measured by the potential drops across R_2, because these potential drops were equal to those across R_3 under balanced conditions. The measurements of electrode resistances were made on the electrodes when in contact with Ringer's solution by comparing the calibrating potentials recorded with the input of the 'voltage' amplifier when connected with *A* and *B* respectively.

Microelectrodes

In order to allow strong currents to flow through a microelectrode without changing its resistance, microelectrodes having a resistance of 4–8 MΩ had to be selected for use. The tip diameters of such electrodes were of the order of 0·5–1 μ, though no accurate measurements were made. Even with these microelectrodes the resting and action potentials of the impaled axons were kept unchanged for as long as 3 hr or more.

The modified Ringer's solution described in our previous paper was also used (YAMASAKI and NARAHASHI, 1959). All the experiments were conducted at room temperatures ranging from 16°C to 23·5°C.

RESULTS

Voltage-current relation

When a square pulse of polarizing current was applied to an axon through the impaled microelectrode, an electrotonic potential with slow rise and fall developed across the nerve membrane. A series of records is shown in Fig. 2. When weak currents were employed, catelectrotonic and anelectrotonic potentials evoked by equal intensity were quite symmetrical. The rise time of the anelectrotonic potentials became longer and the magnitude of their steady state became greater as the current intensity was raised. With very strong anodal currents, the electrotonic potentials decreased after reaching a maximum showing a hump. At the end of a strong anodal current, the anelectrotonic potential decayed with a phase of slight depolarization. No break excitation occurred with the current

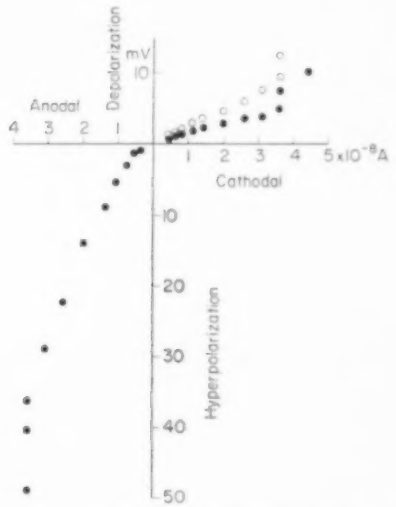


FIG. 3. The relation between the intensity of applied current (abscissa) and the displacement of membrane potential (ordinate). Solid circles indicate the measurements at the final steady amplitude, while hollow circles indicate those at the initial hump. Axon H in Table 1.

intensities used. On the other hand, the catelectrotonic potentials did not grow so markedly as the current intensity was raised; they reached a steady state of low magnitude after forming a hump. When the hump of the catelectrotonic potential reached a critical level, an action potential was generated from the summit of this hump. No repetitive excitation was initiated by raising the current intensity as high as twice to three times the threshold. At the end of a strong cathodal current, the catelectrotonic potential decayed rapidly with a phase of hyperpolarization.

A typical example of the relation between applied currents and electrotonic potentials is illustrated by Fig. 3. The magnitudes of the catelectrotonic potentials

VOL. 3 1959

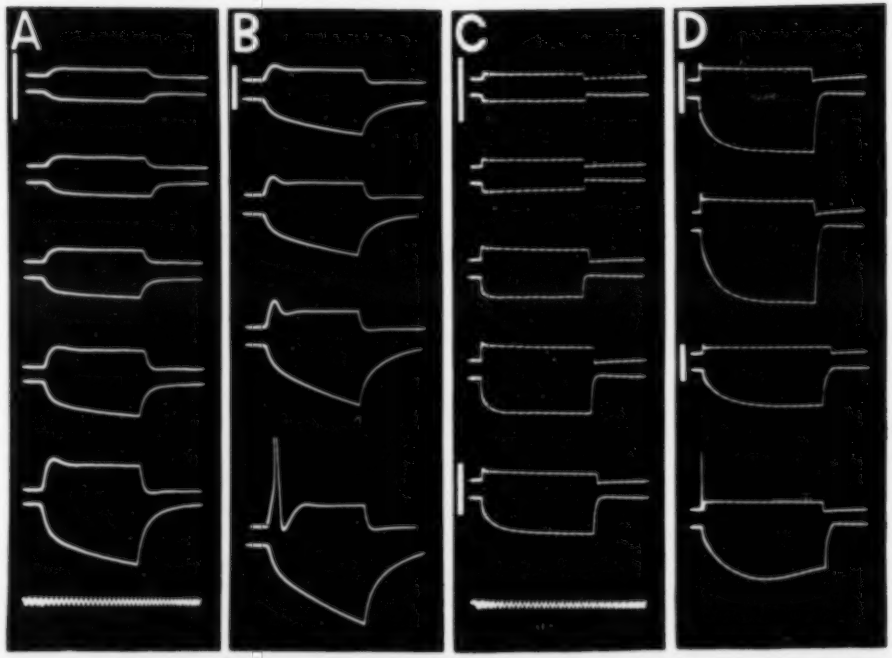


FIG. 2. Electrotonic potentials produced by square pulses of polarizing current. Each pair of records indicates a catelectrotonic (upward deflexion) and an anelectrotonic potentials (downward deflexion) evoked by equal intensity of current. (A), from top to bottom, current intensities are 3·52, 5·68, 7·49, 11·8 and $20·2 \times 10^{-9}$ A. Voltage calibration, 10 mV. A time marker of 1 kc/sec applies to both (A) and (B). (B), current, 27·5, 35·2, 37·8 and $45·3 \times 10^{-9}$ A. Voltage, 20 mV. The action potential in the bottom pair is truncated. (C), as in (A), but with slower sweep. Current, 3·86, 5·56, 7·83, 10·9 and $20·2 \times 10^{-9}$ A. An upper voltage calibration of 10 mV applies to the top to the fourth pair, while a lower one of 20 mV to the bottom pair. A time marker of 100 c/sec applies to both (C) and (D). (D), as in (B), but with slower sweep. Current, 25·7, 30·8, 36·2 and $43·8 \times 10^{-9}$ A. An upper voltage calibration of 20 mV applies to the top and the second, while a lower one of 50 mV to the third and the bottom. The action potential in the bottom is truncated. Axon H in Table 1.

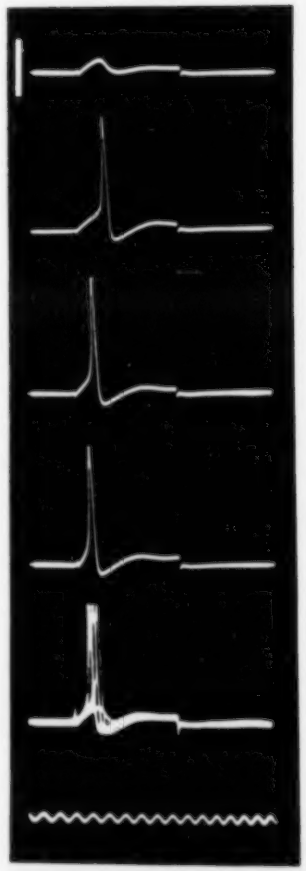


FIG. 5. Responses of the axon evoked by various intensities of cathodal current. Current intensities are, from the top to the fourth record, 4·86 (subthreshold), 5·02 (threshold), 5·32 and $6·54 \times 10^{-8}$ A. The bottom shows superimposed records with current intensities varying from subthreshold to suprathreshold. Voltage calibration, 50 mV. Time marker, 1 kc/sec. Axon B in Table 1.

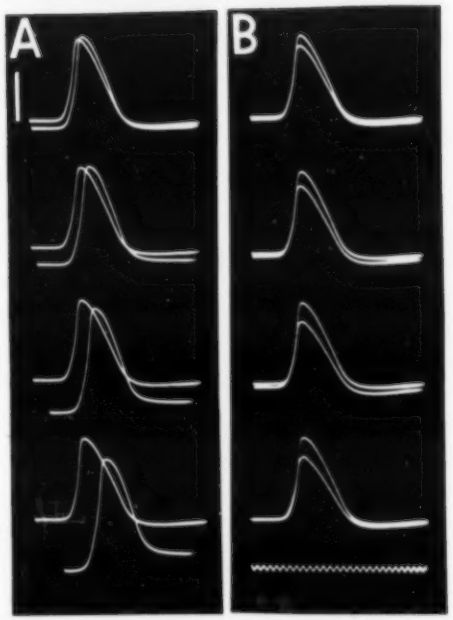


FIG. 7. Action potentials under electrotonus. (A), under anelectrotonus. Intensities of polarizing current are, from top to bottom, 2·52, 3·51, 4·71 and $5·85 \times 10^{-8}$ A. A voltage calibration of 50 mV applies to both (A) and (B). (B), as in (A), but under catelectrotonus. A time marker of 10 kc/sec applies to both (A) and (B). Axon B in Table 1.

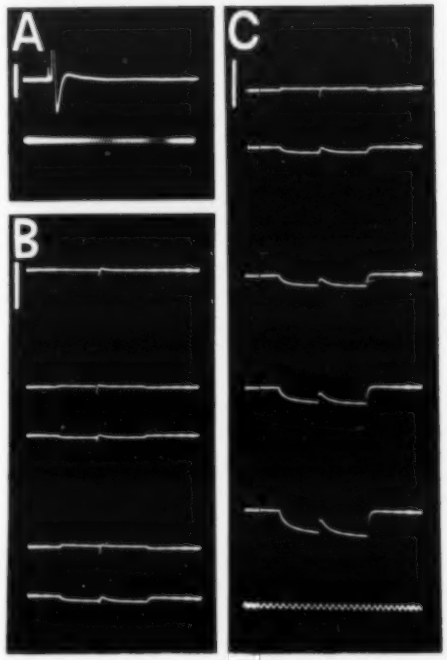


FIG. 9. After-potentials under electrotonus. Spike potentials are too faint to be reproduced. (A), no polarizing current. Voltage calibration, 5 mV. Time marker, 1 kc/sec. (B), from top to bottom, 0, 6·32 (cathodal and anodal) and $11·1 \times 10^{-9}$ A (cathodal and anodal). Voltage calibration, 50 mV. (C), from top to bottom, 13·2 (cathodal and anodal), 34·9 (anodal), 41·0 (anodal) and $49·4 \times 10^{-9}$ A (anodal). Voltage calibration, 50 mV. A time marker of 100 c/sec applies to both (B) and (C). Axon B in Table 1.

were measured both at the summit of hump and at the steady state, but the anelectrotonic potentials were measured at only the steady state because the hump was not observed in the anelectrotonic potentials except for very strong currents. The voltage-current relation clearly indicates that the membrane resistance increases as the intensity of anodal current is raised, while it decreases as the intensity of cathodal current is raised (cf. Fig. 6). The rectification with cathodal current may be called 'delayed rectification' as HODGKIN *et al.* (1949) have called it.

Local response

A square pulse of cathodal current produced a hump in the course of electrotonic potential if it was of sufficient intensity. Superimposing the records of electrotonic potentials produced by equal and opposite currents revealed the fact that the catelectrotonic potential is composed of an electrotonic potential superimposed with a small negative potential (Fig. 4). This small potential

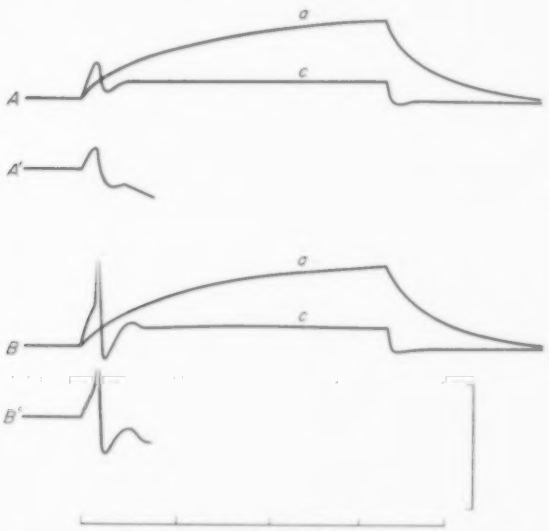


FIG. 4. *A*, superimposed tracings of the catelectrotonic, *c*, and anelectrotonic, *a*, potentials produced by equal intensity of subthreshold current. *A'*, a subtracted tracing from *c* to *a*. *B* and *B'*, as in *A* and *A'*, but with threshold current. The action potential is truncated. Voltage calibration, 50 mV. Time marker, 10 msec intervals. Axon B in Table 1.

increased in magnitude as the current intensity was raised, and it generated an action potential when it reached a critical size. It is undoubtedly a local or graded response in the region of the cathode, which has generally been observed with other nerves and muscles (e.g. HODGKIN, 1938, 1948, 1951; KATZ, 1937, 1947). It should be noted that the subtracted tracings in Fig. 4 undoubtedly do not show the true magnitude and time course of the local response. This view is

based on the following reasons: with the current intensities used, the membrane resistance is so increased by hyperpolarization that the potential level at any instant must be greater than that expected from the resting membrane resistance and the current intensity. On the other hand, the membrane resistance is so decreased with delay by depolarization that the catelectrotonic potential must be smaller than that expected from the resting membrane resistance and the current intensity except for the initial phase. It follows that the magnitude of the apparent local potential so obtained is certainly somewhat smaller than the true magnitude, and that the apparent positive phase that follows a negative local potential is somewhat overestimated.

Critical depolarization and threshold current

The values of the critical depolarization from which an action potential is generated are shown in Table 1 together with the intensities of threshold current. These gave a mean value of 22 mV for critical depolarization and $4{\cdot}42 \times 10^{-8}$ A for threshold current. Responses of the axon depolarized by a variety of current intensities are shown in Fig. 5. It can be seen that the critical depolarization is independent of the current intensity and hence is independent of the time to reach it.

Electrical constants

Electrotonic potentials evoked by square pulses of polarizing current were analysed on the basis of the cable theory introduced by HODGKIN and RUSHTON (1946).

The values of the effective resistance, R_e, were in the range of 207–452 KΩ with a mean of 283 KΩ (Table 1). In order to calculate the specific membrane resistance, R_m, from R_e, it is necessary to know both the specific axoplasm resistance, R_i, and the axon diameter. The axoplasm resistance could not be estimated by recording the electrotonic potential at only zero distance. There is general agreement that the axoplasm resistance is about 2–3 times the specific resistance of the surrounding medium, R_o. The mean value of R_i/R_o was calculated as 2·72 (from Table 3 in WEIDMANN, 1957). The haemolymph serum of the cockroach is found to be isotonic to 224 mM NaCl solution (LUDWIG *et al.*, 1957). The specific resistance of 224 mM NaCl was estimated to be 48 Ω cm by extrapolating the relation between resistances and NaCl concentrations (from table on p. 1546 in HODGMAN, 1939). Hence R_i was assumed to be 130 Ω cm. The diameter of the axon, being the largest one in the nerve cord, was about 50 μ. The values of R_m fell in the range of 410–1940 Ω cm² with a mean of 800 Ω cm² (Table 1). As expected from the voltage-current relation, R_m varied with current intensities, being increased by anodal current and decreased by cathodal current (Fig. 6).

The values of the space constant, λ, fell in the range of 0·63–1·36 mm with a mean of 0·86 mm (Table 1).

The specific membrane capacity, C_m, was in the range of 1·2–12·6 μF cm^{-2} with a mean of 6·3 μF cm^{-2} (Table 1), and was independent of the intensity of

anodal current (Fig. 6). The mean value for the membrane time constant, τ_m, was 4·2 msec with a range of values from 1·6 to 7·7 msec (Table 1).

TABLE 1—ELECTRICAL PROPERTIES OF THE COCKROACH GIANT AXONS

Axon	Temperature (°C)	R_e (KΩ)	R_m (Ωcm²)	C_m (μF cm^{-2})	τ_m (msec)	λ (mm)	Critical depolarization (mV)	Threshold current ($\times 10^{-6}$ A)
A	23·5	217	450	8·7	3·9	0·65	—	—
B	23	252	600	8·3	5·0	0·76	18	5·02
	23	300	850	9·1	7·7	0·91	—	—
C	20	273	710	2·2	1·6	0·82	23	4·26
	20	452	1940	1·2	2·3	1·36	—	—
D	21·5	301	860	3·0	2·6	0·91	—	—
E	21·5	359	1230	3·3	4·1	1·09	29	3·19
F	22	207	410	12·6	5·2	0·63	17	4·56
	22	262	650	11·2	7·3	0·79	—	—
G	22	264	660	6·5	4·3	0·80	—	—
H	16	277	730	3·0	2·2	0·84	15	4·53
	16	232	510	7·0	3·6	0·70	—	—
I	16	—	—	—	—	—	26	5·74
J	16	—	—	—	—	—	24	3·62
Mean	20	283	800	6·3	4·2	0·86	22	4·42

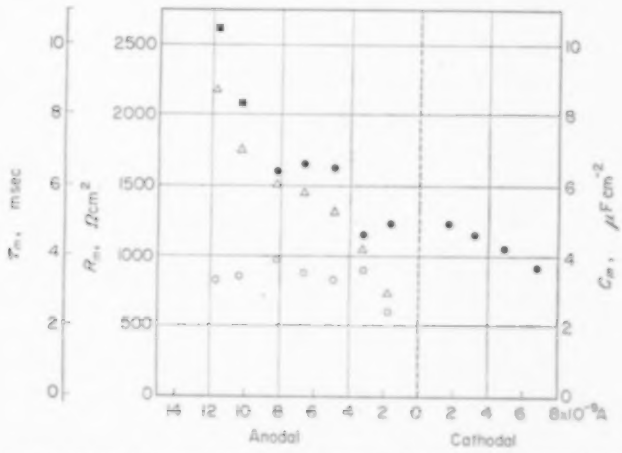


FIG. 6. The relations between the applied current (abscissa) and membrane resistance (R_m, solid circles), time constant (τ_m, triangles) and membrane capacity (C_m, hollow circles) (ordinate). Axon E in Table 1.

Relation between membrane potential and action potential

There is general agreement that the amplitude of the action potential decreases under catelectrotonus while it increases under anelectrotonus (e.g. COOMBS *et al.*,

1955; HAGIWARA and WATANABE, 1954; ITO, 1957; WEIDMANN, 1955). The cockroach giant axon has also been shown to behave similarly (CORABOEUF and BOISTEL, 1955). Furthermore, there is convincing evidence that the rate of rise of the action potential is largely dependent on the membrane potential, being increased by raising the membrane potential and decreased by lowering the membrane potential (ITO, 1957; WEIDMANN, 1955).

An action potential, which was evoked by a single shock delivered through a pair of external electrodes, was sent to the region where a polarizing current was being applied through the impaled microelectrode. One example of a series of

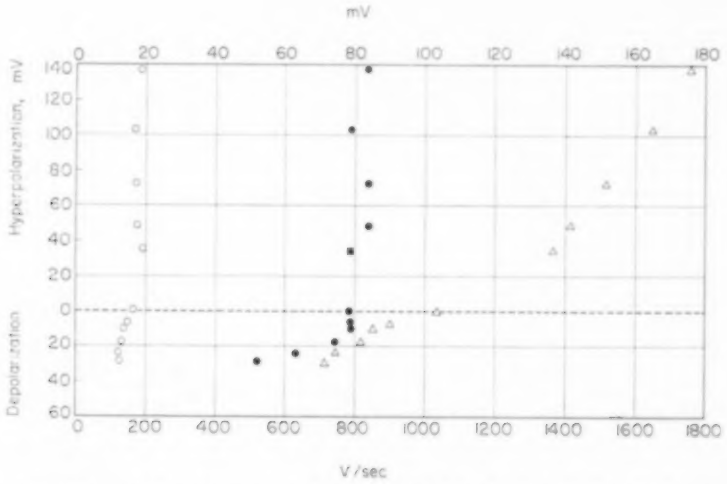


FIG. 8. The relation between action potential (abscissa) and the displacement of membrane potential (ordinate). Triangles indicate the magnitudes, solid circles the rates of rise and hollow circles the rates of fall of the action potential. Axon C in Table 1.

records is shown in Fig. 7. The magnitude, the maximum rate of rise, and the maximum rate of fall of the action potential are plotted against the displacements of the membrane potential as shown in Fig. 8. It can be seen that the magnitude of the action potential is largely dependent on the membrane potential. With weak currents of either direction the changes in spike height came close to compensating for the displacements of the membrane potential, whereas with stronger currents the changes in spike height could not compensate sufficiently for the displacements. An extrapolation of the curve relating the spike height to the membrane potential revealed that the spike height would not become zero even when the membrane was depolarized completely. Such a relation suggests that the action potential is generated not by a non-selective ion sink, as in the end-plate region, but by a selective permeability change to one species of ions (HAGIWARA and WATANABE, 1954).

The maximum rates of rise and fall of the action potential underwent little change by hyperpolarizing the membrane. On the other hand, they were decreased to some extent by depolarization (Figs. 7 and 8).

Relation between membrane potential and after-potential

There is general agreement that the magnitude of the after-positivity is dependent upon the membrane potential, being increased by depolarization and decreased by hyperpolarization (ARAKI and OTANI, 1955; COOMBS *et al.*, 1955; EYZAGUIRRE and KUFFLER, 1955; ITO, 1957). Furthermore, when the anodal current is further increased, the after-positivity inverts into after-negativity (ITO, 1957). In the cockroach giant axons, the spike potential was followed by an after-positivity of small amplitude and the latter in turn was followed by an after-negativity of much smaller amplitude (Fig. 9).

VOL. 3 1959

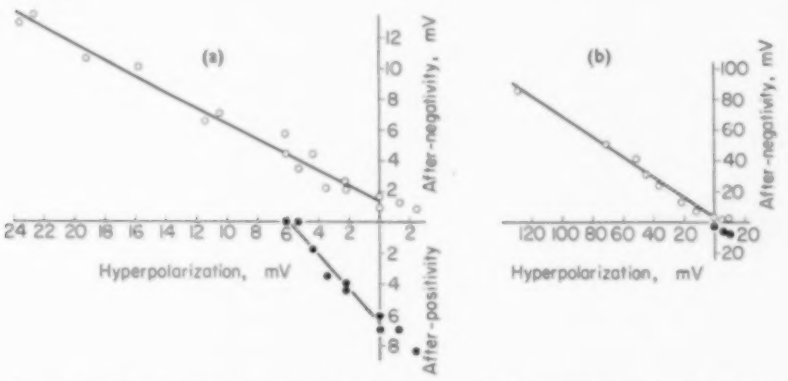


FIG. 10. The relations between the displacement of membrane potential (abscissa) and after-potential (ordinate). Hollow circles indicate the magnitudes of the negative after-potential and solid circles the positive after-potential. (a), Axon B in Table 1. (b), Axon C, but with more extended hyperpolarization.

The nerve cord was stimulated by external electrodes while the impaled axon was being polarized. One example of a series of records is shown in Fig. 9. It was found that the after-positivity was increased in magnitude by depolarization whereas it was decreased by hyperpolarization. It eventually disappeared when the hyperpolarization exceeded a certain level. The after-negativity was decreased in magnitude by depolarization and increased by hyperpolarization. Plotting the magnitudes of the positive and negative after-potentials against the displacements of the membrane potential resulted in two separate straight lines as illustrated by Fig. 10. It can be seen that the straight line for the after-positivity has a slope of about unity under hyperpolarization whereas the slope for the after-negativity is less than unity. It follows from these observations that the after-positivity and the after-negativity are independent of each other.

Ionic currents associated with an action potential

According to HODGKIN and KATZ (1949) the total quantity of sodium entering the axon during an impulse is given by $C_m V/F$, where V is the potential difference across the nerve membrane (in the case of action potential, the magnitude of action potential), and F is the Faraday. In the cockroach giant axons, C_m is estimated to be 6·3 μF cm^{-2} and the magnitude of the action potential 95 mV (YAMASAKI and NARAHASHI, 1959). Hence the total quantity of sodium entering the axon is $6{\cdot}2 \times 10^{-12}$ M cm^{-2} impulse^{-1}. However, the total quantity of sodium which enters should be larger than $C_m V/F$ because (1) sodium entry during the rising phase may be partly neutralized by exit of potassium or entry of chloride, and (2) sodium entry may continue during the falling phase (HODGKIN, 1951). The sodium entry is calculated as $1{\cdot}4$–$1{\cdot}6 \times 10^{-12}$ M cm^{-2} impulse^{-1} for *Loligo* giant axons and $1{\cdot}55 \times 10^{-12}$ M cm^{-2} impulse^{-1} for *Sepia* giant axons (HODGKIN, 1951; HODGKIN and KATZ, 1949; KEYNES and LEWIS, 1951). In fact, however, the observed values of the sodium entry are more than twice the calculated ones, being $3{\cdot}5 \times 10^{-12}$ M cm^{-2} impulse^{-1} for *Loligo* axons and $3{\cdot}7$–$3{\cdot}8 \times 10^{-12}$ M cm^{-2} impulse^{-1} for *Sepia* axons (KEYNES, 1951; KEYNES and LEWIS, 1951). If the similar relation were assumed for the cockroach giant axons, the sodium entry would be as large as about $1{\cdot}5 \times 10^{-11}$ M cm^{-2} impulse^{-1}.

The ionic current entering the axon at the moment when the rate of rise of the action potential is at a maximum is given by:

$$I_i = -C_m \frac{\partial V}{\partial t},$$

where I_i is the net inward current per cm^2 due to transfer of ions from outside to inside (HODGKIN and KATZ, 1949). The maximum rate of rise of the action potential is estimated to be 1,100 V sec^{-1}, and the maximum rate of fall 380 V sec^{-1} (YAMASAKI and NARAHASHI, 1959). Hence the net ionic inward current during the rising phase is 6·9 mA cm^{-2}, and the net ionic outward current during the falling phase is 2·4 mA cm^{-2}. The actual inward current must be even greater, because a large passive outward current is presumably flowing through the depolarized membrane at that instant (FATT and KATZ, 1953).

The inward current during the rising phase of the action potential is estimated to be 0·6–1·0 mA cm^{-2} for squid giant axons (*Loligo*) (HODGKIN *et al.*, 1949, 1952; HODGKIN and KATZ, 1949), 1·29 mA cm^{-2} for crab muscle (*Portunus* and *Carcinus*) (FATT and KATZ, 1953), and 2 mA cm^{-2} for frog muscle (NASTUK and HODGKIN, 1950). Therefore, the value of 6·9 mA cm^{-2} for the cockroach giant axons is considerably greater than those of other nerves and muscles.

DISCUSSION

Our previous study, in which the effects of varying the external sodium and potassium concentrations on the resting and action potentials from the cockroach giant axon have been examined, provides convincing evidence that the sodium theory (HODGKIN, 1951) is also applicable to this axon (YAMASAKI and NARAHASHI,

1957). Based on this theory the depolarization causes a transient increase in membrane conductance to sodium which is followed by a slower but maintained increase in potassium conductance (HODGKIN and HUXLEY, 1952a, b; HODGKIN *et al.*, 1949, 1952). The rectification observed with cathodal current is therefore explained in terms of the increase in potassium permeability, and the delay in terms of the time required for the potassium permeability to change.

The after-positivity following a spike potential in the roach axons is explicable by assuming that the increased potassium permeability during the falling phase of the action potential is maintained for some time after the spike, because the resting potential is smaller than the potassium equilibrium potential (YAMASAKI and NARAHASHI, 1959). The phase of hyperpolarization following a catelectrotonic potential would then be directly analogous to the after-positivity following an action potential. A positive phase is also found to follow a local potential set up by a subthreshold current in the roach axons. It should be noted that the presence of such a positive phase which follows the subthreshold response is demonstrated by calculation based on the sodium theory (HODGKIN and HUXLEY, 1952b).

VOL. 3 1959

TABLE 2—ELECTRICAL CONSTANTS OF NERVES AND MUSCLES

Tissue	Fibre diameter (μ)	R_m (Ωcm^2)	R_i (Ωcm)	C_m ($\mu F\ cm^{-2}$)	τ_m (msec)	λ (mm)	Reference
Squid giant axon (*Loligo*)	500	700	30	1·0	0·7	6·0	COLE and HODGKIN (1939); HODKIN *et al.* (1949)
Squid giant axon (*Sepia*)	200	9200	60	1·2	14	5·7	WEIDMANN (1951)
Crab axon (*Carcinus*)	30	7700	90	1·1	6·8	2·0	HODGKIN (1947)
Lobster axon (*Homarus*)	75	2300	60	1·3	2·3	1·6	HODGKIN and RUSHTON (1946)
Frog muscle (*Rana*)	45	4300	230	5	19	1·1	KATZ (1948)
Crab muscle (*Portunus* and *Carcinus*)	180	120	70	42	4·6	0·9	FATT and KATZ (1953)
Kid Purkinje fibre	75	1900	110	12	20	1·9	WEIDMANN (1952)

The dependence of the after-positivity following a spike upon membrane potential strongly supports the explanation for the after-positivity production mentioned above. It seems likely that the mechanism responsible for the after-negativity production is different from that for the after-positivity, because the slopes for both after-potentials are independent of each other as illustrated by Fig. 10. This view is also supported by the finding that a small dip is observed between the spike potential and the after-negativity even when the after-positivity disappeared by hyperpolarization.

Some of the electrical constants so far obtained are listed in Table 2. It will be seen that the membrane resistance of the roach giant axon is comparable to that of *Loligo* giant axon, but that the membrane capacity is considerably high as compared with other axons. A high membrane capacity, together with a relatively high rate of rise of the action potential (YAMASAKI and NARAHASHI, 1959), calls for an intense exchange of ions during excitation as calculated in this paper. In connexion with a great value for the rate of rise of the action potential, it is of interest to find the ineffectiveness of hyperpolarization on the rate of rise. This finding appears to be inconsistent with the experiment on Purkinje fibres in which an S-shaped curve relating the rate of rise to the membrane potential is obtained (WEIDMANN, 1955). The discrepancy, however, would be explained if it were assumed that in the roach axon the rate of rise of the action potential generated at the normal resting membrane corresponds to the upper limiting value of the S-shaped curve. In other words the sodium carrier system in the roach axon is assumed to be in an activated condition at the normal resting potential.

SUMMARY

The electrical properties of the cockroach giant axons have been examined by means of intracellular microelectrodes. One and the same microelectrode was used for stimulating and recording by employing a Wheatstone bridge circuit.

A delayed rectification was observed when the intensity of cathodal current exceeded a certain level. Furthermore a local potential, which was followed by an after-positivity, was set up by a subthreshold cathodal current. The membrane resistance was increased by raising the intensity of anodal current. With a very strong anodal current, the electrotonic potential decreased after reaching a maximum showing a hump.

The mean critical depolarization was 22 mV, and the mean threshold current was $4{\cdot}4 \times 10^{-8}$ A.

The electrical constants were as follows: specific membrane resistance 800 Ω cm^2, specific membrane capacity 6·3 μF cm^{-2}, time constant 4·2 msec and space constant 0·86 mm.

The magnitude of the action potential was increased by hyperpolarization and decreased by depolarization.

The maximum rate of rise of the action potential underwent little change by hyperpolarization, but was decreased by depolarization.

The after-positivity was increased by depolarization and decreased by hyperpolarization. The after-negativity was decreased by depolarization and increased by hyperpolarization. A straight line relating the after-positivity to the membrane potential had a slope of about unity under hyperpolarization, whereas a straight line for after-negativity had a slope of less than unity under hyperpolarization.

The calculated values of $6{\cdot}2 \times 10^{-12}$ M cm^{-2} impulse^{-1} and 6·9 mA cm^{-2} were obtained for the total quantity of sodium entering the axon during an impulse

and for the net ionic inward current during the rising phase of an action potential, respectively.

The nature of the delayed rectification, the mechanisms of the after-positivity and the after-negativity productions, and the peculiarities of the electrical properties in the roach giant axon are discussed.

Acknowledgement—We are greatly indebted to Dr. S. HAGIWARA for his kind criticism during the course of this work.

REFERENCES

ARAKI T. and OTANI T. (1955) Response of single motoneurones to direct stimulation in toad's spinal cord. *J. Neurophysiol.* **18**, 472–485.

BOISTEL J. and CORABOEUF E. (1954) Potential de membrane et potentiels d'action de nerf d'insecte recueillis à l'aide de microélectrodes intracellulaires. *C.R. Acad. Sci. Paris* **238**, 2116–2118.

COLE K. S. and HODGKIN A. L. (1939) Membrane and protoplasm resistance in the squid giant axon. *J. gen. Physiol.* **22**, 671–687.

COOMBS J. S., ECCLES J. C., and FATT P. (1955) The electrical properties of the motoneurone membrane. *J. Physiol.* **130**, 291–325.

CORABOEUF E. and BOISTEL J. (1955) Quelques aspects de la microphysiologie nerveuse chez les insectes. *Colloq. int. Cent. nat. Rech. sci.* **67**, 57–72.

EYZAGUIRRE C. and KUFFLER S. W. (1955) Further study of soma, dendrite, and axon excitation in single neurons. *J. gen. Physiol.* **39**, 121–153.

FATT P. and KATZ B. (1953) The electrical properties of crustacean muscle fibres. *J. Physiol.* **120**, 171–204.

FRANK K. and FUORTES M. G. F. (1956) Stimulation of spinal motoneurones with intracellular electrodes *J. Physiol.* **134**, 451–470.

HAGIWARA S. and WATANABE A. (1954) Action potential of insect muscle examined with intra-cellular electrode. *Jap. J. Physiol.* **4**, 65–78.

HODGKIN A. L. (1938) The subthreshold potentials in a crustacean nerve fibre. *Proc. roy. Soc.* (B) **126**, 87–121.

HODGKIN A. L. (1947) The membrane resistance of a non-medullated nerve fibre. *J. Physiol.* **106**, 305–318.

HODGKIN A. L. (1948) The local electric changes associated with repetitive action in a non-medullated axon. *J. Physiol.* **107**, 165–181.

HODGKIN A. L. (1951) The ionic basis of electrical activity in nerve and muscle. *Biol. Rev.* **26**, 339–409.

HODGKIN A. L. and HUXLEY A. F. (1952a) Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. *J. Physiol.* **116**, 449–472.

HODGKIN A. L. and HUXLEY A. F. (1952b) A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.* **117**, 500–544.

HODGKIN A. L. and KATZ B. (1949) The effect of sodium ions on the electrical activity of the giant axon of the squid. *J. Physiol.* **108**, 37–77.

HODGKIN A. L. and RUSHTON W. A. H. (1946) The electrical constants of a crustacean nerve fibre. *Proc. roy. Soc.* (B) **133**, 444–479.

HODGKIN A. L., HUXLEY A. F., and KATZ B. (1949) Ionic currents underlying activity in the giant axon of the squid. *Arch. Sci. physiol.* **3**, 129–150.

HODGKIN A. L., HUXLEY A. F., and KATZ B. (1952) Measurement of current-voltage relations in the membrane of the giant axon of *Loligo*. *J. Physiol.* **116**, 424–448.

HODGMAN C. D. (1939) *Handbook of chemistry and physics*. Chemical Rubber Publishing Co. Ohio.

VOL. 3 1959

ITO M. (1957) The electrical activity of spinal ganglion cells investigated with intracellular microelectrodes. *Jap. J. Physiol.* **7**, 297–323.

KATZ B. (1937) Experimental evidence for a non-conducted response of nerve to subthreshold stimulation. *Proc. roy. Soc.* (B) **124**, 244–276.

KATZ B. (1947) Subthreshold potentials in medullated nerve. *J. Physiol.* **106**, 66–79.

KATZ B. (1948) The electrical properties of the muscle fibre membrane. *Proc. roy. Soc.* (B) **135**, 506–534.

KEYNES R. D. (1951) The ionic movements during nervous activity. *J. Physiol.* **114**, 119–150.

KEYNES R. D. and LEWIS P. R. (1951) The sodium and potassium content of cephalopod nerve fibres. *J. Physiol.* **114**, 151–182.

LUDWIG D., TRACEY S. K. M. and BURNS M. (1957) Ratios of ions required to maintain the heart beat of the American cockroach, *Periplaneta americana* Linnaeus. *Ann. ent. Soc. Amer.* **50**, 244–246.

NASTUK W. L. and HODGKIN A. L. (1950) The electrical activity of single muscle fibres. *J. cell. comp. Physiol.* **35**, 39–74.

WEIDMANN S. (1951) Electrical characteristics of *Sepia* axons. *J. Physiol.* **114**, 372–381.

WEIDMANN S. (1952) The electrical constants of Purkinje fibres. *J. Physiol.* **118**, 348–360.

WEIDMANN S. (1955) The effect of the cardiac membrane potential on the rapid availability of the sodium-carrying system. *J. Physiol.* **127**, 213–224.

WEIDMANN S. (1957) *Elektrophysiologie der Herzmuskelfaser.* Medizinischer Verlag Hans Huber, Bern und Stuttgart.

YAMASAKI T. and NARAHASHI T. (1957) Studies on the mechanism of action of insecticides (XIV). Intracellular microelectrode recordings of resting and action potentials from the insect axon and the effects of DDT on the action potential. *Botyu-Kagaku* **22**, 305–313.

YAMASAKI T. and NARAHASHI T. (1959) The effects of potassium and sodium ions on the resting and action potentials of the cockroach giant axon. *J. Ins. Physiol.* **3**, 146–158.

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 243 *to* 255. *Pergamon Press Ltd., London. Printed in Great Britain*

THE SALIVARY SECRETIONS OF A PLANT-SUCKING BUG, *ONCOPELTUS FASCIATUS* (DALL.) (HETEROPTERA: LYGAEIDAE)—I

THE TYPES OF SECRETION AND THEIR ROLES DURING FEEDING

P. W. MILES*

Department of Zoology, University of Cambridge

(*Received* 25 *November* 1958)

Abstract—*Oncopeltus* secretes two types of saliva: sheath-material, which coagulates rapidly and forms a lining to the path of the stylets when the insect feeds on natural food materials; and a watery and water-soluble saliva, which is secreted and sucked back again both on the surfaces of substrates and within them. *Dysdercus fasciatus* Sign. (Pyrrhocoridae) secretes both types of saliva in a similar manner. In agar gels containing sucrose or homogenized peanut, *Oncopeltus* deposits watery saliva, some of which is sucked back several minutes after secretion. During this process, substances are leached from the medium and starch grains are broken down by an amylase. The course of feeding in both natural and artificial media is described. It is suggested that, once feeding has begun, the secretion of watery saliva is inhibited by the flow of liquids up the food-canal; that stylet-activity is inhibited by the presence of acceptable substances in these liquids, but cessation of the inhibition can be brought about by sensory adaptation; and that the secretion of sheath-material is elicited by resistance to the passage of the stylets, but the rate of flow is uncontrolled and only a limited amount can be secreted continuously. The functions of the salivary secretions are considered, with reference to the feeding of the phytophagous Hemiptera generally.

VOL. 3 1959

INTRODUCTION

In a previous paper (Miles, 1958b), reference was made to two types of salivary secretion produced by Heteroptera: the stylet-sheath material which sets rapidly to form a 'track' lining the path of the stylets during the penetration of food materials; and a watery secretion which, *inter alia*, is secreted onto surfaces and sucked back into contact with a gustatory organ when the insect is exploring substrates with the tip of the rostrum. The present paper describes observations on the secretion of both types of saliva during feeding, with special reference to the milkweed bug, *Oncopeltus fasciatus* (Dall.), and discusses the significance of the secretions in the feeding processes of Hemiptera.

THE CULTURE OF THE INSECTS AND TREATMENT OF SUBSTRATES

The milkweed bugs used in this study were reared on milkweed seed and water, the latter forming an essential part of their diet. *Dysdercus fasciatus* Sign. (Pyrrhocoridae), on which some observations were also made, was reared similarly on soaked cotton-seed and water.

* Now at the Waite Agricultural Research Institute, University of Adelaide.

To study the feeding of *Oncopeltus* on plant materials, either sections of milkweed stem (about 4 mm in diameter and 20 mm long) or freshly blanched peanuts were placed in the insects' rearing dishes for 24–48 hr. The material was then fixed in Carnoy's or Petrunkewitsch's solution, and paraffin sections 10 μ thick were cut and stained in safranin and light green or in Fleming's triple stain.

The artificial substrates mentioned in this paper were essentially those described by MILES (1958a). Nutrients were added to a 2·5 per cent agar dispersion in water, and the mixture was cooled to 50°C and poured on to cold microscope slides to form films about 1 mm thick. After the gel had set, the slides were placed film-uppermost among the insects, either in their rearing dishes or, to permit the observation of the course of feeding under the microscope, in 4 in. glass crystallizing basins in which adults or last instar larvae were confined.

It was found, towards the end of this investigation, that when 2·5 per cent gelatine was added to the agar media, the films dried out more slowly and remained attractive to the insects over longer periods.

EVIDENCE FOR THE SALIVARY NATURE OF THE SECRETIONS

When the drinking water with which *Oncopeltus* or *Dysdercus* was provided contained trypan blue, the contents of the midgut became coloured with the dye, whereas both types of secretion described in this paper as 'salivary' remained colourless and were produced in normal quantities. The possibility that either was regurgitated from the midgut was thus eliminated.

In a further paper in this series, evidence will be presented that the contents of the different lobes of the salivary glands correspond in their properties to one or other of the secretions.

FEEDING IN NATURAL SUBSTRATES

In milkweed stems, *Oncopeltus* left stylet-tracks similar to those produced by other phytophagous Hemiptera (SMITH, 1926; PAINTER, 1928). On the surface of stems, as on other food materials, the insect first secreted an external 'collar' of sheath-material (Figs. 1 and 4), similar to one described by SMITH in the case of the aphid *Myzus circumflexis* Buckt. The thin, tubular 'neck' of the collar was formed by *Oncopeltus* within the labial groove and remained enclosed by the rostrum throughout feeding. The stylet-tracks were mainly intracellular and terminated in the vascular bundles. Usually there was extensive branching at the level of the outer ring of phloem, and both phloem and xylem vessels became blocked with sheath-material (Fig. 1).

In peanuts, sheath-material was discharged as a relatively thick mass initially but did not accompany the stylets for the full length of their penetration. This became evident when the material was fixed with the insects' stylets *in situ* by the method of SMITH (1926). Sections showed empty cells surrounding the stylet-path, although many of the starch grains which normally filled the cells were too large to be accommodated unchanged by the food-canal of the stylets (Fig. 2). To investigate this last point further, the insects were dissected at various intervals after they had begun to feed on peanuts, and the contents of the gut were treated with iodine solution; at no time could starch grains be discovered, and it was concluded that the starch which the insects removed from the peanuts was digested extra-intestinally.

VOL.
3
1959

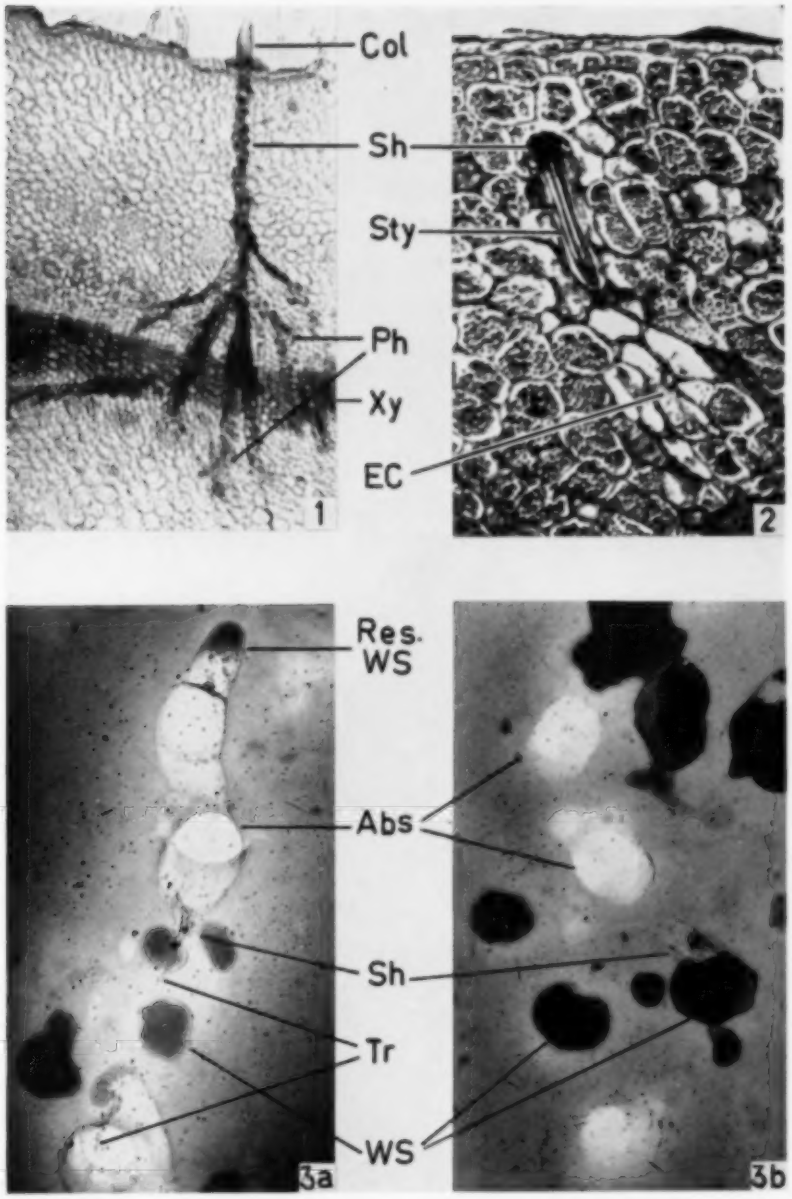


FIG. 1. Hand-cut section of milkweed stem showing the stylet-sheath (Sh) and the external collar of sheath-material (Col) produced by *Oncopeltus*. The tracks end in the phloem bundles (Ph) on either side of the xylem (Xy).

FIG. 2. Microtome section of a peanut showing the regions of empty cells (EC) which result from the feeding of *Oncopeltus* and a fragment of the stylets (Sty) *in situ*. The stylets are surrounded at the proximal end by a thick safranin-stained mass of sheath material (Sh) which diminishes towards the centre of the peanut and is absent from the region of empty cells. Starch grains fill the other cells.

FIG. 3. Films of sucrose-agar gel on which *Oncopeltus* has fed, fixed in formol and stained (a) with safranin and light green and (b) with toluidine blue. Abs, areas where the gel has been absorbed; Res. WS, partially resorbed watery saliva; Sh, mounds of sheath-material; Tr, partially formed 'track' of sheath-material; WS, deposits of watery saliva.

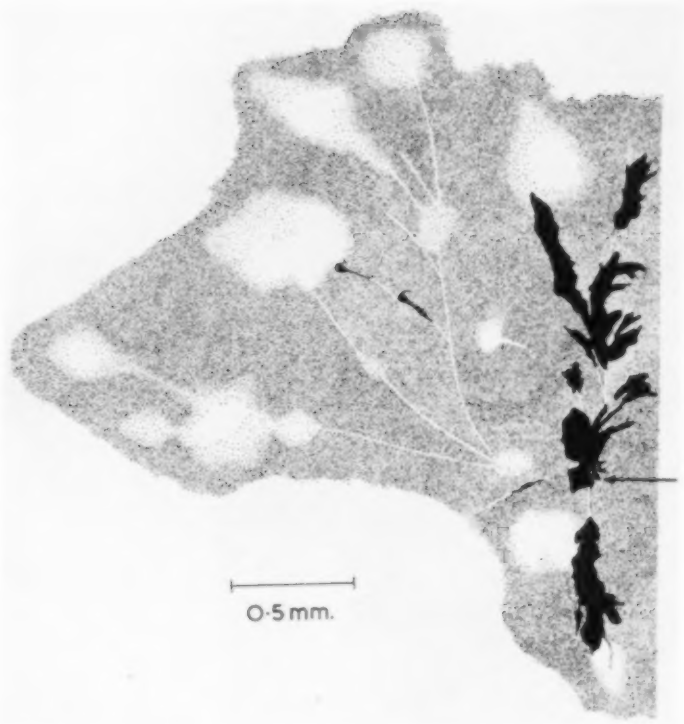


FIG. 5. Camera-lucida drawing, looking down on the surface of a film of sucrose-agar containing Chinese black, showing the absorption produced by the feeding of *Oncopeltus*. The right hand margin is one edge of the film, and the arrow indicates the point of entry of the stylets. The black masses represent sheath-material.

THE SECRETION OF SHEATH MATERIAL IN ARTIFICIAL SUBSTRATES

When *Oncopeltus* inserted its stylets into a sucrose-agar gel, a stylet-sheath was seldom produced. However, the insect would often attempt to pierce the glass slide below the film of gel, and this activity was invariably accompanied by the discharge of drops of a viscous secretion which set rapidly and cohered to enclose the stylets. In the course of several minutes, a cone-shaped mound of the secretion would then be formed (Fig. 4), both by expansion of the outer layers as more material was secreted within the mound and by material seeping from the base of the mound or from splits or faults in its walls, while rhythmic movements of the head and rostrum indicated continuous stylet-activity within the mound. Eventually, the insect would either abandon its attempts to feed, or the stylets would bend at right angles against the glass and emerge from the base of the mound to become active in the body of the medium, as described by MILES (1958a). Occasionally a stylet-track continuous with the mound of solidified secretion was subsequently formed, indicating that the latter was sheath-material and represented a considerable length of stylet-track that would have been produced had the stylets been moving through a suitable medium.

VOL. 3 1959

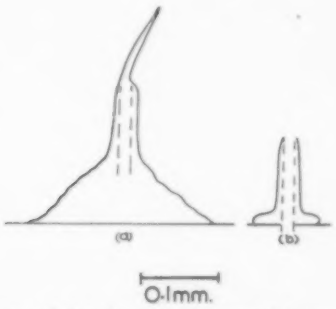


FIG. 4. Outlines of collars of sheath-material secreted (a) onto glass, and (b) at the surface of a milkweed stem.

When the insect withdrew its rostrum from a mound of sheath-material, a tubular neck of the material was revealed, similar to that found on the external collar secreted on the surfaces of natural substrates. However, here the neck was extended on one side into a filament, which was probably formed by material forced up round the stylets and farther into the labial groove by the pressure generated within the mound during its formation.

DEPOSITION OF A WATER-SOLUBLE SECRETION IN ARTIFICIAL SUBSTRATES

When slides coated with 2·5 per cent agar containing 10 per cent sucrose were placed amongst either *Oncopeltus* or *Dysdercus* and were subsequently fixed in neutral formol, deposition of another type of salivary secretion, distinct from the

sheath-material, became apparent on staining (Fig. 3). With safranin and light green, the secretion appeared as safranin-staining reticulations of definite outline, except where continuous with an area of total absorption of the substrate by the insect. Here the reticulations became broken and dispersed, consistent with the partial resorption of the secretion. When the slides were immersed for 5 min in 0·1 per cent toluidine blue and dehydrated by passing through alcohol, the reticulations appeared dark blue on a ground-colour of purple within the boundary of the reticulations; and when the slides were air dried after staining and passed straight to xylene for clearing, the masses were finely granular and everywhere purple.

The material was never present in slides on which the insects had not attempted to feed, and it was always deposited within the film. It could not be demonstrated in unfixed slides by any of the staining methods described above, indicating that the secretion was soluble in water; nor could it be found after immersion of the slides in Carnoy's solution. Further, the masses corresponded in shape and size to areas of the substrate absorbed by the insect.

In sucrose-agar substrates, the discharge of watery liquid was occasionally visible at the moment when the stylets first emerged from the base of a mound of sheath-material secreted onto the glass beneath; and the emission of a watery secretion could also be observed if the stylets broke the surface during their movement through the medium; but otherwise the secretion of the water-soluble material was difficult to observe directly, since the refractive indices of the secretion and substrate were too similar. However, when 5 per cent wet weight of homogenized peanut was added to 2·5 per cent agar, the resulting medium, on which the insects fed readily, permitted direct observation of the secretion into it of both types of saliva. It was observed that in such a substrate *Oncopeltus* would make several stylet-channels, which were initially left filled with a watery liquid, and that eventually the stylets would begin to return to previously made channels and suck back their contents; and since holes pricked in the medium with a fine pin always remained empty, it was clear that the liquid initially left in the channels was secreted by the insect. All the channels made at one feeding remained open throughout, and columns of liquid left in them could be observed moving backwards and forwards as the insect's stylets made corresponding movements in some other part of the system.

ABSORPTION OF NUTRIENTS FROM ARTIFICIAL SUBSTRATES

When *Oncopeltus* fed on a sucrose-agar gel which contained a fine suspension of Chinese black, removal of the particles indicated where materials were absorbed from the substrate and also left a record of the paths taken by the stylets (Fig. 5). It was found that the clear areas did not always coincide with a breakdown of the physical structure of the substrate and therefore that, in sucrose-agar media, total absorption or disruption of the substrate gave an inadequate indication of the absorption of nutrients (see Fig. 3). It was also confirmed that absorption could take place far removed from deposits of sheath-material.

To determine the absorption of different components from a complex food source, peanut-agar slides were given to *Oncopeltus* and the following techniques were used to demonstrate the food materials remaining:—

Protein. The slides were immersed in Millon's reagent overnight at 20°C. Protein in the film stained orange-red, and the sheath-material a brilliant cherry-red.

Oils and Fats. A saturated solution of Sudan Black B or Sudan III in 70 per cent alcohol was used to reveal oil droplets. The sheath-material stained slightly with both.

Starch. The slides were immersed overnight in lactophenol containing 0·01 per cent iodine. Starch grains stained purple and the sheath-material yellow.

In peanut-agar substrates, absorption always produced partial breakdown of the structure of the medium, accompanied by the removal of protein and starch. Oil droplets of the order of 10 μ in diameter were dispersed throughout the medium, but in areas of general absorption, large droplets up to 0·1 mm across were found, and material which stained strongly with the Sudan stains was occasionally found in the central canal of mounds of sheath-material. It was noted, as before, that many of the starch grains in the substrate were either larger than the food-canal of the insect's stylets, or of the same order of diameter, and that breakdown of the grains appeared to be essential before they could be removed from the substrate by the insect.

VOL. 3 1959

SEPARATE COLLECTION OF THE SALIVARY SECRETIONS

To collect sheath-material in quantity and free of other substances, saturated sucrose solution was poured onto a microscope slide and left to dry and harden. When crystals had begun to form, the slide was placed in the insects' rearing dish for 24 hr; the sucrose was then washed away leaving mounds of sheath-material cemented to the slide.

It was not found possible to extract the watery secretion which had been discharged within agar substrates; and although a watery saliva is also secreted by the insects against any surface with which the tip of the rostrum makes contact (Miles, 1958b), after about 0·5 mm³ had been accumulated by placing the rostrum in a capillary tube, the insect would suck the secretion back again. However, it was found that the discharge of watery saliva was elicited and augmented when chemoreceptors on the fore tarsi and tips of the antennae were stimulated with dilute acetic acid, and if, at the same time, the insect was secured with the rostrum extended, a capillary tube could then be used to take up the drops as they exuded. The secretion thus obtained did not coagulate, was soluble in water, and was shown to contain amylase by the method of Pickford and Dorris (1934). There was thus no reason to suppose that it differed from the watery and water-soluble saliva which was secreted within agar media and appeared to break down starch grains.

DISCUSSION

The nature of the salivary secretions of Hemiptera

Early writers (e.g. Petri, 1908, 1909; Smith, 1926; Painter, 1928) generally believed that the stylet-sheath associated with the feeding of phytophagous

Hemiptera was precipitated from the contents of the plant cells by the insects' saliva, and this view permitted the assumption that the secretion was of the watery type normally associated with salivary action. However, once observations had been made on the feeding of the insects on artificial substrates, it became apparent that the stylet-sheath was produced solely by a salivary secretion which coagulated rapidly as it left the stylets (Fife, 1932; Bennett, 1934). Such a secretion seems an unlikely vehicle for the digestive enzymes known to be elaborated in the salivary glands of Hemiptera (Zweigelt, 1915; Baptist, 1941; Nuorteva, 1954); yet Storey (1939) was certain that only coagulable material issued from the stylets of the Jassid, *Cicadulina mbila* Naude, and that a water-soluble substance diffused into liquids from the gelling mass at the moment of ejection; and although Day *et al.* (1952) believed that two different salivary secretions were produced by another Jassid, *Orosius argentatus* (Evans), they were unable to demonstrate this conclusively. Capsids unquestionably secrete a watery saliva, since some of this is deposited on the surface of the host plant (Smith, 1920). But these insects produce no stylet-sheath and their manner of feeding is atypical of the phytophagous Heteroptera generally (Goodchild, 1952); and although Smith (1926) described a material, deposited by Capsids in their host plants, which stained similarly to the sheath-material of other Hemiptera, he did not recognize it as a separate salivary secretion, and Goodchild made no mention of such a substance in his comprehensive work on the feeding of cacao Capsids.

In a previous paper (Miles, 1958b), it was reported that various Heteroptera dab surfaces with a watery saliva which is immediately sucked back again; and the present work has demonstrated that, in *Oncopeltus* and *Dysdercus*, this secretion is distinct from stylet-sheath material, and that the watery saliva is also ejected within substrates which the stylets have penetrated. These results make it probable that at least most of the phytophagous Heteroptera produce both salivary materials as distinct secretions and are in keeping with the observations of Day *et al.* on Jassids within the Homoptera.

The secretion of saliva by Oncopeltus

The watery saliva secreted by *Oncopeltus* and *Dysdercus* during feeding could have a simple leaching function in such a medium as a sucrose-agar gel, but in the case of *Oncopeltus* feeding on peanut, the secretion also seems capable of the extra-intestinal digestion of starch. In either capacity, it is unlikely that the secretion could act instantaneously, and Baptist (1941) even concluded that the salivary amylases of Hemiptera were too slow-acting to effect the extra-intestinal digestion of starch. It is therefore significant that *Oncopeltus*, in sucrose-agar and peanut-agar, leaves deposits of watery saliva, some of which are sucked back later.

Nevertheless, there is no evidence that deposits of watery saliva are left in milkweed stems, and observations on the feeding of *Oncopeltus* on sucrose solutions suggest that the insect may modify its method of extracting nutrients from different media according to their nature. Thus when the rostrum is in contact

with a saturated sucrose syrup, the secretion and sucking back of watery saliva can be readily observed, whereas when the rostrum is in a dilute sucrose solution which contains visible particles (e.g. of Chinese black), these show an uninterrupted streaming of liquid into the stylets. It is possible to reconcile these observations by assuming that during feeding the flow of liquids up the food-canal inhibits the ejection of watery saliva, which is thus secreted only when the medium is solid, or too viscous, or contains too high a proportion of particles which cannot be accommodated by the canal; and this assumption may incidentally explain the apparent discrepancy between the observations of STOREY and of DAY *et al.* on the nature of the salivary secretions of Jassids.

On the other hand, if the secretion of watery saliva is always accompanied by sucking activity, it is necessary to consider how deposits of the secretion accumulate in food materials. It seems unlikely that the watery saliva is always secreted at a greater rate than it can be sucked back, but two other possibilities exist. In the first place, watery saliva may diffuse into the surrounding food material so that it cannot be reclaimed by the insect until the material is rendered more fluid by the action of the secretion or, in the case of agar gels, until prolonged stylet-activity results in the disruption of the medium. Secondly, when the stylets have forced a passage in a medium which is of such a consistency that the stylet-channel does not collapse when the stylets are withdrawn, or when the collapse of the channel is prevented by the presence of a stylet-sheath, the insect may not be able to exert sufficient suction to remove the watery saliva secreted into the channel during withdrawal of the stylets. This explanation would meet the conditions found in peanut-agar gels, in which it has been observed that watery saliva is left in the stylet-channels initially. Further, retraction of the stylets in this and other agar media has been observed to take place in two ways; during the early stages of stylet-activity, retractions are gradual and characterized by small backward and forward 'pumping movements' of the stylets, whereas in the later stages, retractions are usually rapid, continuous backward movements (MILES, 1958a). It is therefore reasonable to suppose that during the first, gradual withdrawal of the stylets from a newly-made channel, saliva is secreted into it; that when the stylets later return to the channel, the contents are ingested as they are displaced by the stylets; and that during the final, continuous withdrawal, even if watery saliva were secreted, little would be left behind because of the rapid movement of the stylets.

In *Oncopeltus*, the secretion of sheath-material and the form of the sheath are also dependent on the nature of the medium with which the stylets make contact. When the insect attempts to pierce glass, a mound of sheath-material accumulates; when it feeds on peanuts, the sheath is thick but does not accompany the stylets for the full length of their penetration; when it feeds on milkweed stems, the sheath is of the type normally associated with hemipterous feeding, viz.: a relatively thin tube enclosing the entire stylet-path; but a sheath is rarely produced in agar gels, and never in liquids. A possible explanation of these observations is that the secretion of sheath-material is elicited by resistance to

VOL.
3
1959

the passage of the stylets, but that the rate of flow of the secretion is uncontrolled and once the supply stored in the lumen of the gland is discharged, some time must elapse before an appreciable quantity is again available. However, it must be noted that *Oncopeltus* and *Dysdercus* differ from many Homoptera in not secreting a stylet-sheath in agar gel and liquids, and it may be that the resistance of water alone to the movement of the stylets is sufficient to elicit the secretion of sheath-material by the smaller phytophagous Hemiptera.

The control of stylet-activity

There is a close connexion between salivary function and stylet-activity in *Oncopeltus*. The pattern of movement of the stylets determines whether deposits of watery saliva will accumulate, where they will accumulate, and whether they are subsequently resorbed; and the secretion of sheath-material occurs only during stylet-activity. No experiments were made to determine how stylet-movement is elicited, but certain inferences, of value in elucidating salivary function in natural food materials, can be drawn from observations on the movement of the stylets in homogeneous media.

It has been observed that a starved insect will sometimes force its stylets into polythene tubing for several minutes and to depths greater than 1 mm if chemoreceptors on the tarsi are stimulated by an adjacent drop of sucrose solution, and if the rostrum when it is swung down makes contact with the polythene. When this happens, movements of the head and rostrum indicate that stylet-activity is continuous until the stylets are eventually withdrawn. Movement of the stylets is also extensive and almost continuous in sucrose-agar gel, but when the insect has pushed its stylets into saturated sucrose, no movement occurs for several minutes. Since it is unlikely that the insect can obtain any acceptable substances within polythene, contact with which will not alone elicit the feeding response, it appears that stylet-activity can occur in the absence of stimulation of the gustatory organ internal to the stylets. Whereas the fact that stylet-movement does not at first occur in saturated sucrose suggests that inhibition of stylet-activity may result from stimulation of the gustatory organ by acceptable substances. The movement of the stylets which can eventually be observed in saturated sucrose could then be attributed to adaptation of the gustatory organ, and this explanation would also cover the continuance of stylet-movement once the stylets have penetrated a sucrose-agar gel, since the insect first spends several minutes leaching sucrose from the surface by dabbing it with watery saliva which is then sucked back. To account for the initiation of stylet-activity, it is only necessary to presume that, in the absence of inhibition, stylet-movement either automatically follows the extension of the rostrum, or that it is a separate stage in the feeding reaction which is elicited by stimulation of sensilla, e.g. at the tip of the rostrum.

Dethier *et al.* (1956) have shown that the time taken for the gustatory organs of the blowfly to become adapted to sucrose solutions increases with increasing concentration, and it may be objected that when *Oncopeltus* feeds on saturated

sucrose, the gustatory organ internal to the stylets would be unlikely to become adapted before the insect has ingested sufficient to bring about the cessation of feeding. However, there is no reason to believe that the concentration of sucrose absorbed by *Oncopeltus* from saturated sucrose is particularly high, since the sugar is picked up in watery saliva which is sucked back immediately after it is ejected.

DETHIER *et al.* (1956) suggested that feeding in the blowfly continues only as long as sensory input from the chemoreceptors is maintained, and that feeding ceases when these organs become adapted. But adaptation of one set of gustatory organs in the blowfly does not cause the cessation of feeding if other organs continue to supply the necessary input. *Oncopeltus* will penetrate polythene and will also make a prolonged attempt to pierce a glass slide below a film of sucrose-agar gel when the coagulating sheath-material rapidly insulates the stylets from any external source of acceptable substances; hence it follows that if continuous sensory input from chemoreceptors is required to maintain feeding in *Oncopeltus*, such input can originate from sensilla other than those internal to the stylets, e.g. from the tarsal chemoreceptors or possibly from chemoreceptors at the tip of the rostrum. Thus, in the absence of sensory input from the gustatory organ internal to the stylets, feeding would cease only when the external chemoreceptors became adapted to the stimuli which initiated feeding.

VOL. 3 1959

It was previously suggested (MILES, 1958b) that Heteroptera select the point at which they insert their stylets into a substrate by 'sampling' the substances picked up by watery saliva which is dabbed on to the surface and is sucked back again. However, if stylet-movement occurs only in the absence of sensory input from the gustatory organ internal to the stylets, the interesting possibility arises that the 'sampling' of surfaces continues until the gustatory organ becomes adapted, and that only then do the stylets begin to penetrate the medium. An incidental observation, that *Oncopeltus* after prolonged starvation (5–7 days) will attempt to pierce clean glass or polythene with little preparatory 'sampling', but will dab the surface of sucrose-agar gel for long periods before inserting its stylets, lends further support to this view.

When stylet-movement does occur, it is evident from the tracks left in milkweed stems and from the pattern of absorption in peanuts and artificial substrates, that the tip of the bundle tends to progress straight through the medium before it probes from side to side in a region near the limit of penetration; and that, if feeding continues, the stylets are then withdrawn for some distance and advance again in a new direction.

The course of feeding in different media

Comprehensive explanations of the course of feeding by *Oncopeltus* in the various media in which this has been observed can be put forward if the inferences outlined above are utilized; viz.—that, during feeding, the secretion of watery saliva is inhibited by the flow of liquids up the stylet food-canal; that the sheath-material is secreted when there is resistance to the passage of the stylets, but the rate of

flow is uncontrolled and only a limited amount can be secreted continuously; and that stylet-movement is inhibited by the presence of acceptable substances in the liquids sucked up the food canal, but the inhibition may cease due to adaptation of the gustatory organ involved.

When the insect inserts its stylets into a peanut, watery saliva would be secreted since the cell contents are not sufficiently fluid to be sucked back in quantity; and if it is assumed that the gustatory organ internal to the stylets rapidly becomes adapted to the concentrations of acceptable substances which can be sucked back at this stage, while sensory input from the external chemoreceptors is maintained, the stylets would continue to advance into the medium. The hardness of the material may be expected to make the progress of the stylets relatively slow, and this would account for the production of the short, thick sheath, which ends before the stylets have completed their penetration and presumably because the insect's immediate supply of sheath-material is exhausted. As the stylet-bundle probes from side to side near the limit of its penetration, the tip would eventually come into contact with material rendered fluid by the action of the watery saliva. Stylet-movement and the secretion of saliva would then cease while this material was ingested. It is unlikely that adaptation to such concentrated and complex food material would occur for some time, and since any further movement of the stylet-bundle would be likely to bring the tip into contact with more, partially digested cell-contents in adjacent regions, the area of empty cells seen in microtome sections would eventually result (Fig. 2). Finally, feeding would stop and the stylets would be withdrawn when sensory input from all the chemoreceptors, both external and internal to the stylets, ceased through adaptation.

In milkweed stems, the stylets apparently penetrate more quickly, and a thin and extensive stylet-sheath is formed. Watery saliva may be secreted as the stylets move through the parenchyma, but if progress is relatively rapid, no deposits would accumulate. When the stylets penetrate phloem vessels, since phloem sap is sufficiently fluid to be taken up the stylet food-canal unchanged (Mittler, 1957), stylet-movement and the secretion of saliva would cease until the supply of sap diminished. The stylets would then move on to tap another phloem vessel, at the same time either extending or branching the stylet-track to produce the pattern of tracks seen in sections (Fig. 1).

In sucrose-agar gel, if the gustatory organ internal to the stylets becomes adapted to the concentration of sucrose sucked back during the initial exploration of the surface, this would explain the extensive stylet-movement observed once penetration begins. The medium itself does not apparently offer sufficient resistance to the stylets to elicit the secretion of sheath-material and a typical stylet-sheath is seldom produced, but the whole supply of material may be expended in an attempt to penetrate the slide beneath. The insect is unable to suck liquid directly from the medium, and watery saliva would be secreted continually and diffuse into the medium where the stylets probe from side to side near the limits of their penetration in any direction. Prolonged stylet-action and sucking may

then cause sufficient disruption of the medium to result in the absorption of some areas of the gel (Fig. 3). In peanut-agar, the pattern of events is evidently similar, except that a typical stylet-sheath is more often produced, mostly at the edge of the film where it is drier than elsewhere, presumably because of the thicker consistency of the medium; and the stylet-paths are less extensive, possibly because the medium is a richer source of acceptable substances which inhibit stylet-activity as they are made available by the action of deposits of watery saliva.

When the insect's stylets are in saturated sucrose, the viscosity of the syrup prevents the insect from sucking it back unchanged, and watery saliva would therefore be secreted. Since the insect sucks back the saliva immediately after it is ejected, the concentration of sugar picked up by this means may not be very high; and it has been suggested above that, although the presence of the sugar would at first inhibit stylet-activity, the gustatory organ internal to the stylets may become adapted to the concentration ingested while sensory input from the external chemoreceptors continues, resulting in the stylet-activity which eventually occurs. Mounds of sheath-material would then be secreted where the stylets make contact with the glass surface below the syrup.

VOL. 3 1959

The function of the stylet-sheath

BÜSGEN (1891), who correctly interpreted the stylet-sheath formed during the feeding of aphids as a secretion produced by the insects, suggested that it functioned as a support for the stylets; but MITTLER (1954) pointed out that the sheath is soft enough to allow the stylets to break through during branching of the track; and, in the case of Heteroptera, the suggestion is further discounted by the fact that *Oncopeltus* can penetrate peanut tissue and guide the stylets in agar gel without the aid of a stylet-sheath. ZWEIGELT (1915), who likewise identified the stylet-sheath of aphids as a salivary secretion, considered that it had more than a merely mechanical function. He presented evidence that the insect secreted a salivary 'diastase', and since he did not consider the possibility of there being two salivary secretions, he assigned a digestive function to the sheath itself. However, this would not seem to be tenable at least in the case of the Heteroptera. For other writers, who believed that the sheath was produced by the plant as a reaction to the feeding of sucking insects, the question of the function of the stylet-sheath hardly arose, and few further suggestions have been made. SUKHOV (1944) claimed that the sheath secreted by *Myzus persicae* Sulz. is never open at the end, and that it acts as a filter which prevents viruses from entering the insect; but *Oncopeltus* and *Dysdercus* certainly absorb materials from the open end of the sheath or beyond it.

The most convincing hypothesis of the function of the stylet-sheath is that put forward by MITTLER, who suggested that the stylet-sheath of aphids prevents sap from exuding round the stylets to the exterior, where it would be lost to the insect. Such a suggestion would apply as well to other sap-sucking Hemiptera, and it is likely that, in the same way, the sheath would prevent the escape of

watery saliva injected into the stylet-channels and of food materials rendered liquid by the action of this secretion, especially when pressure is exerted by forward movement of the stylets. Moreover, the existence of an external collar of sheath-material which fits into the labial groove could be expected to make the sheath particularly efficient in preventing losses of this kind.

Acknowledgements—This paper represents part of a thesis submitted for the degree of Ph.D. in the University of Cambridge; and my sincere thanks are due to my supervisor, Professor V. B. WIGGLESWORTH, and to Professor Sir James GRAY for providing laboratory facilities. I am also grateful to Rhodes University, South Africa; to the trustees of the Southern Rhodesian State Lotteries; and to the trustees of the Charelick Salomon Scholarship Fund, Southern Rhodesia, for scholarships and grants which made the work possible.

REFERENCES

BAPTIST B. A. (1941) The morphology and physiology of the salivary glands of Hemiptera-Heteroptera. *Quart. J. micr. Sci.* **82**, 91–139.

BENNETT C. W. (1934) Plant-tissue relations of the sugar-beet curly-top virus. *J. agric. Res.* **48**, 665–701.

BÜSGEN M. (1891) Der Honigtau. Biologische Studien an Pflanzen und Pflanzenläusen. *Z. Naturwiss.* **25**, 340–428.

DAY M. F., IRZYKIEWICZ H., and MCKINNON A. (1952) Observations on the feeding of the virus vector *Orosius argentatus* (Evans), and comparisons with certain other Jassids. *Aust. J. sci. Res.* (B) **5**, 128–142.

DETHIER V. G., EVANS D. R., and RHOADES M. V. (1956) Some factors controlling the ingestion of carbohydrates by the blowfly. *Biol. Bull., Woods Hole* **111**, 204–222.

FIFE J. M. (1932) A method of artificially feeding the sugar-beet leafhopper. *Science* **75**, 465–466.

GOODCHILD A. J. P. (1952) A study of the digestive system of the West African cacao capsid bugs (Hemiptera, Miridae). *Proc. zool. Soc. Lond.* **122**, 543–572.

MILES P. W. (1958a) The stylet movements of a plant-sucking bug, *Oncopeltus fasciatus* Dall. (Heteroptera: Lygaeidae.) *Proc. R. ent. Soc. Lond.* **33** (A), 15–20.

MILES P. W. (1958b) Contact chemoreception in some Heteroptera, including chemoreception internal to the stylet food canal. *J. Ins. Physiol.* **2**, 338–347.

MITTLER T. E. (1954) The feeding and nutrition of aphids. *Thesis, Univ. of Cambridge, England.*

MITTLER T. E. (1957) Studies on the feeding and nutrition of *Tuberolachnus salignus* (Gmelin) (Homoptera, Aphididae) 1. The uptake of phloem sap. *J. exp. Biol.* **34**, 334–341.

NUORTEVA P. (1954) Studies on the salivary enzymes of some bugs injuring wheat kernels. *Ann. ent. fenn.* **20**, 102–124.

PAINTER R. H. (1928) Notes on the injury to plant cells by chinch bug feeding. *Ann. ent. Soc. Amer.* **21**, 232–242.

PETRI L. (1908) Einige Bemerkungen über die Rolle der Milben bei der Dactylopius-Krankheit der Reben. *Zbl. Bakt.* (II) **21**, 375–379.

PETRI L. (1909) Über die Wurzelfäule Phylloxierter Weinstöcke. *Z. Pflkrankh.* **19**, 18–48.

PICKFORD G. E. and DORRIS F. (1934) Micro-methods for the detection of proteases and amylases. *Science* **80**, 317–319.

SMITH K. M. (1920) Investigation of the nature and cause of the damage to plant tissue resulting from the feeding of capsid bugs. *Ann. appl. Biol.* **7**, 40–55.

SMITH K. M. (1926) A comparative study of the feeding methods of certain Hemiptera and of the resulting effects upon the plant tissue, with special reference to the potato plant. *Ann. appl. Biol.* **13**, 109–139.

STOREY H. H. (1939) Investigations of the mechanism of the transmission of plant viruses by insect vectors. III. The insects' saliva. *Proc. roy. Soc.* (B) **127**, 526–543.

SUKHOV K. S. (1944) Salivary secretion of the Aphis *Myzus persicae* (Sulz.) and its ability to form a filtering apparatus. *C. R. Acad. Sci.* **42**, 226–228.

ZWEIGELT F. (1915) Beiträge zur Kenntnis des Saugphänomens der Blattläuse und der Reaktionen der Pflanzenzellen. *Zbl. Bakt.* (II) **42**, 265–335.

VOL.
3
1959

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 256 *to* 261. *Pergamon Press Ltd., London. Printed in Great Britain*

THE EXCRETORY EFFICIENCY OF THE AMERICAN COCKROACH, *PERIPLANETA AMERICANA* L.

R. L. PATTON, J. GARDNER, and ANN D. ANDERSON

Cornell University, Ithaca, New York

(*Received* 28 *November* 1958)

Abstract—The efficiency of the excretory system of the American cockroach was measured using a dye clearance test. Indigo carmine in aqueous solution was injected into the blood and the rate of its removal was checked by densitometric measurements of blood spots on filter paper strips. The results show that the rate of excretion is significantly changed by changes in temperature and that certain biologically active chemicals inhibit the rate of excretion.

INTRODUCTION

EXCRETION is a regulatory function in animals. By the activity of the organ of excretion the composition of the blood and tissue fluids which comprise the internal medium is maintained at a nearly constant level. Any factor that interferes with the excretory mechanism does so at the expense of the well-being of the individual.

The principal organ of excretion of insects is a series of tubular structures called the Malpighian tubes. Their mode of operation has been investigated by perfusion (PATTON and CRAIG, 1939), by observing the ability of the tubes to take up vital dyes (LISON, 1937, 1938), and by cannulation experiments in which the effluent was collected for analysis (RAMSAY, 1952, 1954, 1955, 1956). In addition to the Malpighian tubes it has been shown that parts of the digestive tract have a regulatory function. These are discussed by WATERHOUSE and DAY (1953).

METHODS

In order to measure the excretory efficiency and to evaluate the effects of biologically active chemicals upon the excretory function, it was necessary to devise a method which would include the activity of all excretory systems in the individual and which would be less cumbersome than the techniques already established. The procedure which resulted involves the rate of elimination of a vital dye from the blood. It has precedent in similar methods used for the same purpose in human physiology and medicine. Indigo carmine in a $2\frac{1}{2}$ per cent aqueous solution was injected into the pericardial cavity of the test insect adjacent to the dorsal vessel. Enough colour to the blood was given by $2\frac{1}{2}$–5 μl to make

possible a measurement of the dye concentration with a photoelectric densitometer. Indigo carmine was used in all of the experiments, but there is no technical reason to exclude the use of other vital dyes. Late instar larvae of the American cockroach were selected for all experiments.

The injected insects were held for 30 min to allow the dye to mix with the blood. This period could be shortened materially by physically shaking the insects either on an electric vibrator or manually. In either case the insect was enclosed in a test tube with padded ends. The treatment gave no indication of injury. Dyed blood samples were taken at hourly intervals on strips of Whatman No. 1 filter paper. Each sample was collected directly from a clipped antenna and absorbed in the centre of a black ring stamped on the paper with stamp pad ink using a one hole rubber stopper. The size of the centre (the hole in the stopper) was selected to coincide with the area over which a microlitre of dyed blood would diffuse on the paper. By this method equal amounts of blood were collected without the use of a pipette. The principles of this method of measurement of volumes and an analysis of the reproducibility are described by BUCK, KEISTER, and ZELLE (1950). The amount of dye in the blood sample was determined by measuring the optical density of the dried spots with a photoelectric densitometer. The instrument was altered to include the entire spot in the illuminated field, and the black ring on the filter paper served as a mask to exclude extraneous light. This method of measurement of the quantity of dye proved to be nearly as accurate as measurements made with a spectrophotometer and was much less cumbersome.

VOL. 3 1959

The results of the optical density measurements were plotted against time. This produced a rate curve describing the rate of elimination of the dye from the blood. Under normal conditions approximately 90 per cent of the dye will be removed during the first 5 hr. The first sample of blood drawn from the insect was used as a time zero reading and the densitometer was set to read at an optical density of 0·6 in each case. This made all subsequent readings relative and the rate curves are directly comparable.

Individual variation among insects selected at random from a laboratory colony is high. An investigation of the factors involved indicated that the greatest deviations came from individuals at or near the time of a moult. At this time most of the physiological processes are badly upset. Variation can be reduced significantly by selecting individuals according to the specific gravity of the blood. Insects whose blood specific gravity falls within a predetermined normal range will respond in a uniform manner. Specific gravity was determined quickly by the gradient column technique of Linderstrøm-Lang as described by LOWRY and HUNTER (1945).

When biologically active chemicals were used they were injected into the body cavities of the test insects 24 hr before the dye elimination measurements were made. This allowed the toxicants to exert their effect upon the organ function before the dye elimination test. The level of dosage ranged between LD_0 and LD_{20}. Water insoluble compounds were dissolved in acetone which did not affect the rate of excretion (Table 1).

Factors investigated include temperature, starvation, dehydration (withholding water for several days), hydration (water injected into the blood), and various biologically active chemicals.

RESULTS

(*A*) *The normal rate of excretion of indigo carmine*

Injected insects held at room temperature (between 25° and 28°C) show a regular and reproducible rate curve which describes the excretion of indigo carmine. This is shown in Fig. 1. The mean optical density and standard deviation of samples from 28 insects was $0{\cdot}46 \pm 0{\cdot}06$ at 60 min (T_1), $0{\cdot}40 \pm 0{\cdot}067$ at 120 min (T_2), and $0{\cdot}38 \pm 0{\cdot}063$ at 180 min (T_3, not shown in Fig. 1). The normal

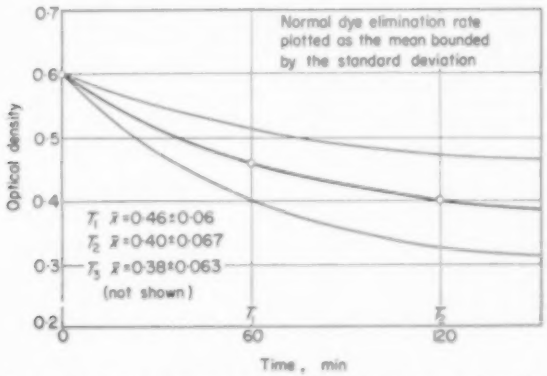


FIG. 1. The rate of excretion of normal American cockroaches.

range is defined by the mean optical density ± the standard deviation. These data have been reproduced by several technicians and by inexperienced students.

(*B*) *The effects of temperature, starvation, dehydration, and excess water*

An increase in temperature usually accelerates the rate of activity in poikilothermic animals and this is true of the rate of excretion. Insects were selected and held at temperatures ranging from 11°C through 40°C at approximately 5° intervals. This conditioning was maintained for 24 hr before the injection of the dye. The temperature was maintained throughout the experiment in each case. The resulting data indicate that the rate of excretion is slowed significantly at the lower temperatures, that it reaches a maximum at about 35°C, and that the rate of excretion is sharply reduced as the temperature is raised higher. The survival rate of the insects at 40°C is low. In Fig. 2 is shown the mean optical densities at 60 min plotted against temperature and the effect of temperature upon the rate of dye clearance.

The factors of hunger, dehydration, and excess water injected at the level of 10–20 per cent of the blood volume estimated on the basis of the weight-volume

relationship of YEAGER and MUNSON (1950) showed no significant change in the rate at which indigo carmine was cleared from the blood.

FIG. 2. The effect of temperature upon the rate of excretion.

(C) *The effects of biologically active chemicals upon excretion*

Selected larvae were injected with sub-lethal doses of a number of chemicals known to be biologically active and after a 24-hr waiting period the excretory function was evaluated. The data were compared to determine the significance with a graphical Z-chart method using the pooled standard deviation and the

TABLE 1—THE COMPARISON OF THE RATES OF EXCRETION BETWEEN CHEMICALLY TREATED AND NORMAL INSECTS

Chemical	Dosage	No. of insects	Pooled standard deviation	Difference between means	Significance of difference
Ethylene glycol	5 μl	29	·077	·09	Significant at 1% level
Acetone	3 μl	5	·063	·05	Not significant
Sodium arsenite	4 μg	11	·08	·03	Not significant
Nicotine	3–5 μg	18	·075	·02	Not significant
Dinitro-*ortho*-cyclo-hexyl-phenol	4 μg	10	·063	·08	Significant at 5% level
DDT	4–8 μg	9	·063	·02	Not significant
*Para*thion	0·12 μg	9	·061	·01	Not significant
Chlordane	1–3 μg	11	·073	·01	Not significant
Aldrin	3–4·5 μg	8	·063	·045	Not significant
Heptachlor	3–4·5 μg	11	·059	·06	Significant at 5% level
Dieldrin	3–4·5 μg	9	·06	·11	Significant at 1% level
*Iso*drin	1·5 μg	14	·076	·13	Significant at 1% level
Endrin	1·5 μg	9	·11	·10	Highly significant at 1% level

difference between the means (SNEDECOR, 1937). These results are summarized in Table 1.

DISCUSSION

These data show that the efficiency of the excretory system of the American cockroach can be evaluated *in vivo* by a relatively simple dye elimination method. Although measurements have been limited to the American cockroach, the technique could be applied to other insects. It also should be possible to extend this method with the use of other vital dyes to assay the activity of sections of the Malpighian system in accordance with the observations of LISON (1937, 1938) that various parts of the tubes absorb dyes depending upon molecular structure and dissociation.

The temperature response curve with a definite maximum and a sharp cut-off as the temperature is raised is interpreted to indicate that the excretory process in insects is secretory and that the system is energized by an oxidative enzyme system. Supporting evidence for this hypothesis is furnished by the observation that the Malpighian tubes of individuals treated with chemicals which inhibit the excretory function (i.e. ethylene glycol) lose their ability to reduce blue tetrazolium (3,3′-dianisole bis 4,4′(3,5-diphenyl) tetrazolium chloride) *in vivo*. This reagent has a reduction potential of about −0·08 V, a correct range for the demonstration of biological oxidation-reduction systems (COLOWICK and KAPLAN, 1957). The failure of the Malpighian tubes to reduce this compound is interpreted to indicate the inhibition of an oxidase system. The observation that DNOCHP, a compound which accelerates oxidative metabolism in all living organisms, significantly increases the rate at which the Malpighian tubes eliminate dye supports this concept.

Very few of the biologically active compounds showed a significant effect upon the rate of excretion, therefore this is a vital function which must be considered in mode of action studies. DIXON (1955) established that ethylene glycol is metabolized by the insect fat body to glycollic aldehyde and glycollic acid and that these are the active inhibitors of Malpighian tube action. Oxalate is not formed and oxalic acid does not show a significant inhibitory effect on excretory function.

Of the insecticides only those of the cyclodiene series show significant activity (Table 1). In ascending order this starts with chlordane followed by aldrin, heptachlor, dieldrin, *iso*drin, and endrin. The latter virtually stops excretory activity and Malpighian tubes of insects treated with endrin are not capable of reducing blue tetrazolium. These observations clearly indicate the need for further study of the enzyme systems in the excretory system as well as those associated with other vital functions as they are affected by biologically active chemicals.

SUMMARY AND CONCLUSIONS

The excretory efficiency of the American cockroach can be measured by a dye clearance method using indigo carmine. The rate of excretion in normal

insects is affected by temperature and shows a definite optimum with a sharp cut-off as the temperature rises. DNOCHP which characteristically accelerates oxidative metabolism increases the rate of excretion. Ethylene glycol and insecticides of the cyclodiene series show the greatest inhibition of the process. Compounds which inhibit the excretory activity also destroy the power of the Malpighian tubes to reduce blue tetrazolium. These data have been interpreted to indicate that excretion in insects is primarily a secretory process which is energized by an oxidative enzyme system which is still to be defined.

REFERENCES

Buck J. B., Keister M. L., and Zelle M. R. (1950) An areal method for calibrating microburettes. *Analyt. chim. acta* **4**, 130–134.

Colowick S. P. and Kaplan H. O. (1957) *Methods in enzymology* **4**, 334. Academic Press, New York.

Dixon S. E. (1955) Studies on the metabolism of ethylene glycol in the fat body and Malpighian tubules of insects. Thesis for the doctorate, Cornell University Library, Ithaca, N.Y.

Lison, L. (1937) Études histophysiologiques sur les tubes des Malpighi des insects. I. L'Elimination des colorants acides chez les Orthoptères. *Arch. Biol., Paris* **48**, 421–460.

Lison L. (1938) Études histophysiologiques sur les tubes des Malpighi des insects. III. L'Elimination des colorants basiques chez les Orthoptères. *Z. Zellforsch.* **28**, 179–209.

Lowry O. H. and Hunter T. H. (1945) The determination of serum protein with a gradient tube. *J. biol. Chem.* **159**, 465–474.

Patton R. L. and Craig R. (1939) The rates of excretion of certain substances by the larvae of the mealworm, *Tebebrio molitor* L. *J. exp. Zool.* **81**, 437–457.

Ramsay J. A. (1952) The excretion of sodium and potassium by the malpighian tubes of *Rhodnius*. *J. exp. Biol.* **29**, 110–126.

Ramsay J. A. (1954) Active transport of water by the malpighian tubes of the stick insect, *Dixippus morosus*. *J. exp. Biol.* **31**, 104–113.

Ramsay J. A. (1955) The excretory system of the stick insect, *Dixippus morosus*. *J. exp. Biol.* **32**, 184–199; 200–216.

Ramsay J. A. (1956) Excretion by the malpighian tubules of the stick insect, *Dixippus morosus*: Calcium, magnesium, chloride, phosphate, and hydrogen ions. *J. exp. Biol.* **33**, 697–709.

Snedecor G. W. (1937) *Statistical methods* pp. 65–66. Collegiate Press, Inc., Ames, Iowa.

Waterhouse D. F. and Day M. F. (1953) Function of the gut in absorption, excretion and intermediary metabolism, 331–349, *in Insect Physiology*, Roeder K. D. John Wiley, New York.

Yeager J. F. and Munson S. C. (1950) Blood volume of the roach *Periplaneta americana* determined by several methods. *Arthropoda* **1**, 255–265.

VOL.
3
1959

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 262 *to* 270. *Pergamon Press Ltd., London. Printed in Great Britain*

A NERVOUS ELEMENT OF UNKNOWN FUNCTION IN THE TYMPANIC ORGANS OF MOTHS*

ASHER E. TREAT†

City College of New York

KENNETH D. ROEDER

Tufts University

(*Received* 28 *November* 1958)

Abstract—A large, richly tracheolated, Type II neurone occurs in the region known as the *Bügel* in the thoracic tympanic organs of many noctuid moths. This cell is the source of continuous and apparently spontaneous impulses in the tympanic nerve. The frequency of these impulses may be reversibly altered by stretching the sheath of tracheal epithelium which surrounds the *Bügel* and encloses the cell together with adjacent portions of the tympanic nerve. Although the B neurone is in close anatomical relation with the nerve fibres coming from the scoloparium, no direct interaction with the acoustic elements has been demonstrated, and the function of the cell remains unknown.

OSCILLOGRAPHIC records of electrical activity in the tympanic organs of noctuid moths usually show a regular and continuous succession of uniform spikes (Fig. 4(a)) somewhat greater in height than those of the acoustic impulses, and quite independent of acoustical stimulation (ROEDER and TREAT, 1957). Similar spikes were recorded by HASKELL and BELTON (1956) from the notodontid *Phalera bucephala* Linn., but were interpreted as representing motor impulses of central origin. That the impulses are actually afferent is shown by their persistence after the nerve is severed from the ganglion. They survive the destruction of the scoloparium which contains the acoustic sensilla, but are extinguished, along with the acoustic responses, when the nerve is divided central to the *Bügel* (*vide infra*), or when the *Bügel* itself is extensively damaged. In a few experiments these non-acoustic impulses have been abolished by minutely localized injury to the *Bügel* without extinguishing the acoustic response.

The facts just stated led us to examine the *Bügel* region histologically. We found there a large Type II neurone which is evidently the source of the non-acoustic impulses. This cell does not appear in the figures or description of the *Bügel* given by EGGERS (1919) or by other writers on tympanic structure. In many respects it resembles the neurone of the stretch receptors found in saturniid moths and other insects by FINLAYSON and LOWENSTEIN (1955, 1958). It does not

* This work was supported in part by grants from the National Science Foundation and the U.S. Public Health Service. Some of the apparatus used was obtained under a previous contract between the Chemical Corps U.S. Army and Tufts University.

† Special Research Fellow of the National Institute of Allergy and Infectious Diseases, 1956–57.

appear to be associated with specific muscles or with other peripheral neurones save those of the acoustic sensilla.

Anatomical relations. The term *Bügel* was used by EGGERS (1919) and by VON KENNEL and EGGERS (1933) to designate two different structures which they regarded as analogous parts of the tympanic organs respectively of noctuid and of geometrid moths.* In both families, the parts in question are apodemal ingrowths of the skeletal frame of the tympanum, serving for the support and

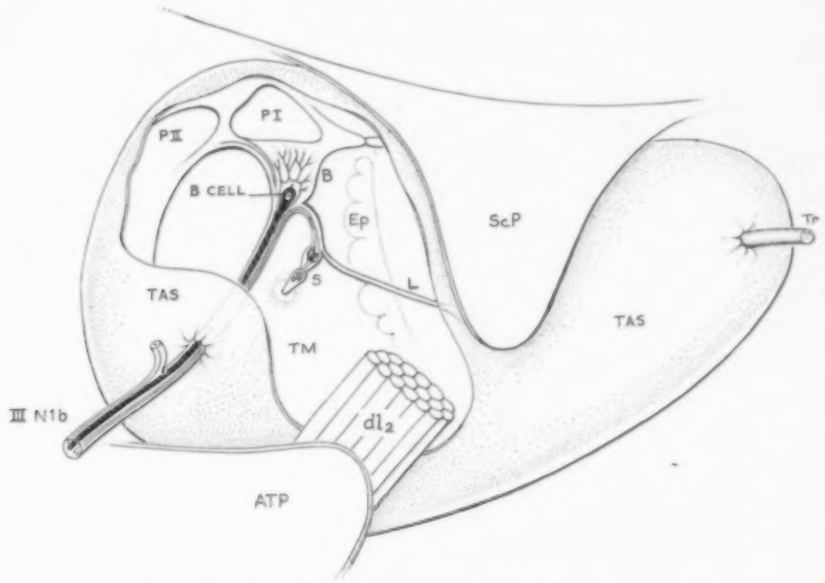


FIG. 1. Schematic drawing of right tympanic air sac and associated structures as seen from within the metathorax. A portion of the air sac is represented as cut away to show the *Bügel*, scoloparium, etc. Cf. photo, Fig. 3c. ATP, anterior tendon plate; B, *Bügel*; dl_2, second dorsolongitudinal muscle of the metathorax; Ep, epaulette or nodular sclerite; L, ligament loosely connecting the scoloparium with the scutal phragma; PI PII, pockets I and II of the tympanic frame; S, scoloparium containing the scolopes and acoustic sensory cells; TAS, tympanic air sac; TM, tympanic membrane; Tr, tracheal twig from metathoracic spiracle; III N1b, tympanic nerve. The countertympanic membrane is not shown.

attachment of one end of the scoloparium and its nerve. Since the structures occur in different segments and are quite different in form and skeletal relationships in the two families, there is no reason to suppose that they are homologous. The geometrid *Bügel* is provided with muscular attachments which may serve to depress it slightly and thereby to alter the tension in the scoloparium, though there is no experimental evidence that such action, if it occurs, has any effect

* The Latin term *stapes* proposed by KIRIAKOFF (*Bull. Ann. Soc. ent. Belg.* **84**, 231–276, 1948) is not widely used and is perhaps too suggestive of a non-existent resemblance to the 'stirrup' of the mammalian ear. Rather than confuse the literature with still another term, we have reluctantly made use of Eggers' *Bügel* (German—stirrup, bent piece, bow).

upon acoustic sensitivity. The noctuid *Bügel* is without direct muscular connexions. Typically it has the form of a lip or flange (Figs. 1, 2, 3c) extending downward into the tympanic air sac from the mouth of pocket I, an internal antrum in the dorsal frame of the tympanic membrane. In some species it is broad and leaf-like; in others it is long, slender, and elastic. It is lined with tracheal epithelium (Fig. 3d) continuous with that which forms the tympanic air sac and

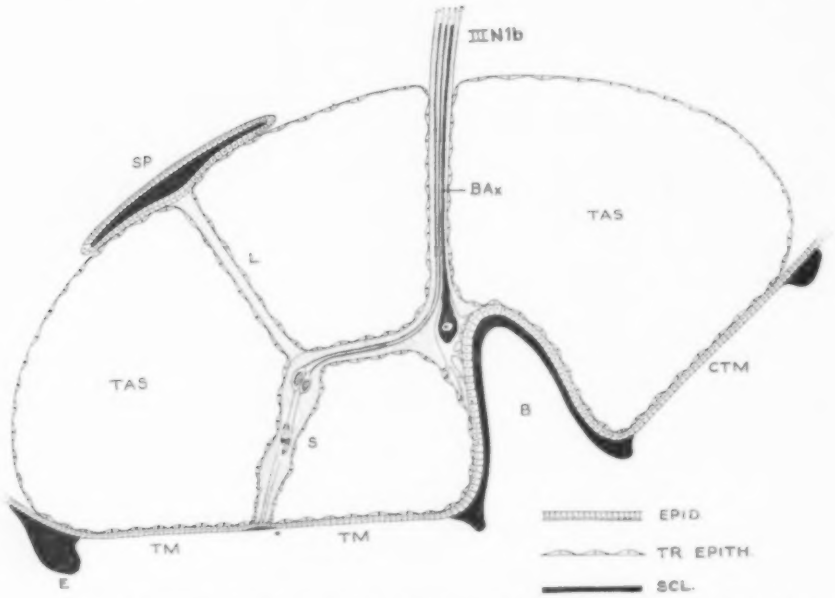


FIG. 2. Schematic frontal section of left tympanic air sac and associated structures, redrawn with modifications from EGGERS (1919). BAx, axon of *Bügel* cell; CTM, countertympanic membrane; EPID, epidermis (shown in embryonic condition to illustrate developmental relation to other structures); TR EPITH, tracheal epithelium; SCL, sclerotization. Other labels as in Fig. 1. Cf. ROEDER and TREAT, 1957.

invests the acoustic scoloparium. This epithelium also extends as a more or less flattened, elastic sleeve from the free tip of the *Bügel* across the lumen of the air sac to its inner, anterior wall. Through this sleeve pass the fibres of the tympanic nerve, including the two fibres from the scoloparium and a large fibre from the non-acoustic neurone (Figs. 1, 2), hereafter called the B neurone or B cell.

The cell body of the B neurone lies between the epithelial covering of the *Bügel* and its skeletal surface, to both of which it is attached by branching tentacular processes (Fig. 3e) which in some cases appear nodular and independently nucleated (Fig. 3f). When the epithelial sheath is stripped from the *Bügel* the neurone comes away with it, much altered in appearance because of the detachment and contraction of these processes. In the region of the neurone (Fig. 3d), the epithelial sheath contains many small, coarsely granular nuclei which often make it difficult

to see the neurone clearly. Rarely it has been possible to strip the sheath from the neurone, revealing a pear-shaped cell body that tapers at one end to a thick axon, and at the other bears a tangle of contracted 'tentacles' (Fig. 3a, b). The structural relations of the cell to the *Bügel* and its sheath make it a difficult object for histological study. Attempts at sectioning have thus far met with little success, and much remains to be learned about the cytological details. For further description see Fig. 3.

The first and some of the best preparations were made by excising the tympanum 20–30 min after intravital injection with 1 per cent methylene blue, and dissecting out the *Bügel* in saline containing a few granules of detergent powder as a wetting agent. Staining of the tympanum after excision, by immersion in detergent saline containing 0·5–1 per cent methylene blue was also occasionally successful. Fixation of methylene blue specimens in saturated ammonium molybdate followed by absolute alchohol gave some fairly good permanent preparations, though not without shrinkage and loss of some features evident in the fresh material. Phase microscopy was an aid to differentiation in both fixed and fresh preparations, though it was difficult to free the specimen sufficiently from extraneous tissue to give the thinness required for good phase contrast.

VOL. 3 1959

The fresh B neurones from *Pseudaletia unipuncta* Haw., ♂, had a cell body measuring approximately 45×20 μ, exclusive of the tentacular process, and tapering to an axon of 14 μ diameter. In a *Macronoctua onusta* Grote, ♀, the cell body when freed from tracheal epithelium measured 20×55 μ, the axon 14 μ. A constant feature of fresh preparations is a rich network of tracheoles ramifying to all parts of the cell body and to the proximal parts of the axon. These become invisible with fixation. In many fresh methylene blue preparations, the central part of the cell contained a large, deeply staining oval body suspended in an envelope of clear fluid which is surrounded in turn by pale blue cytoplasm. Pressure on the coverslip would cause this body to glide to and fro within the clear space. Fixed preparations show a large nucleus in this region.

For permanent preparations various methods other than methylene blue were also tried. Osmic acid (1 per cent) or an osmic acid and platinum chloride (Flemming-Hermann) mixture gave good differentiation after about a half hour's exposure, though with considerable shrinkage. Tentacular structures were usually better preserved in specimens injected with 10 per cent acidified formalin. Silver techniques were unsuccessful, chiefly because of the strong tendency of the tracheal epithelial cell nuclei to take up the stain.

Because of the obscuring effect of the epithelial sheath, it was impossible in most preparations to determine the precise relation of the B neurone to the nerve fibres of the acoustic sensilla, which enter the sheath in close proximity to the B cell body. In a few instances, however, the acoustic fibres could be seen as thin strands that turn just within the sheath and thence run parallel to the much larger B cell axon (Fig. 3a). It would be difficult indeed to destroy the B neurone in a living insect without risk of damage to the acoustic fibres. For this reason, the fact that experimental damage to the B cell has usually been followed by a

diminution or disappearance of the acoustic response does not necessarily indicate a physiological interaction.

Taxonomic distribution. A B neurone has been found in every noctuid species examined. Histological preparations have been made from the following:

Agrotis ypsilon Rott.
Peridroma margaritosa Haw.
Graphiphora c-nigrum Linn.
Pseudaletia unipuncta Haw.
Sunira bicolorago Gn.
Crymodes devastator Brace
Macronoctua onusta Grote
Amphipyra pyramidoides Gn.
Prodenia ornithogalli Gn.
Prodenia eridania, Cram.
Catocala sp.

Physiological evidence for the existence of the B cell has been obtained for most of the species listed above (exceptions: *P. margaritosa* and *P. ornithogalli*) and also for the following noctuids:

Acronycta americana Harr.
Lacinipolia renigera Steph.
Metaxaglaea inulta Grote
Septis arctica Boisd.
Apamea americana Speyer
Papaipema sp.
Autographa precationis Gn.
Catocala, 2 spp.
Zale undularis Dru.
Scoliopteryx libatrix Linn.

Typical B cell activity has been recorded also in the arctiid *Diacrisia virginica* Fabr., in the notodontids *Nadata gibbosa* A. and S. and *Pheosia rimosa* Pack., and in the geometrid *Tetracis crocallata* Gn.*

At the suggestion that the B cell might represent a sensory element having no necessary relation to the acoustic organs as such, and therefore perhaps even more widely distributed among the Lepidoptera, we recorded electrical activity in the

* It is noteworthy that the recordings from the notodontids show acoustic responses in a single fibre only, in contrast to the two fibres evident in the records from Phalaenids. This experience agrees with that of HASKELL and BELTON (1956) with *Phalera*, the genus used by EGGERS (1919) to represent the Notodontidae in his histological studies of the scoloparium. EGGERS was able to find only one scolops in *Phalera*, a circumstance which he explained by the suggestion that a second scolops was hidden by the single one visible in his preparation. From the physiological evidence it appears more likely that the Notodontidae actually possess only a single sensory element in their auditory organs.

The records from *Tetracis crocallata* are believed to be the first from an abdominal tympanic organ in Lepidoptera. They show a degree of complexity consistent with the four scolops known to occur in the geometrid tympanum, but it has not been possible to identify individual spikes as representing particular cells.

VOL.
3
1959

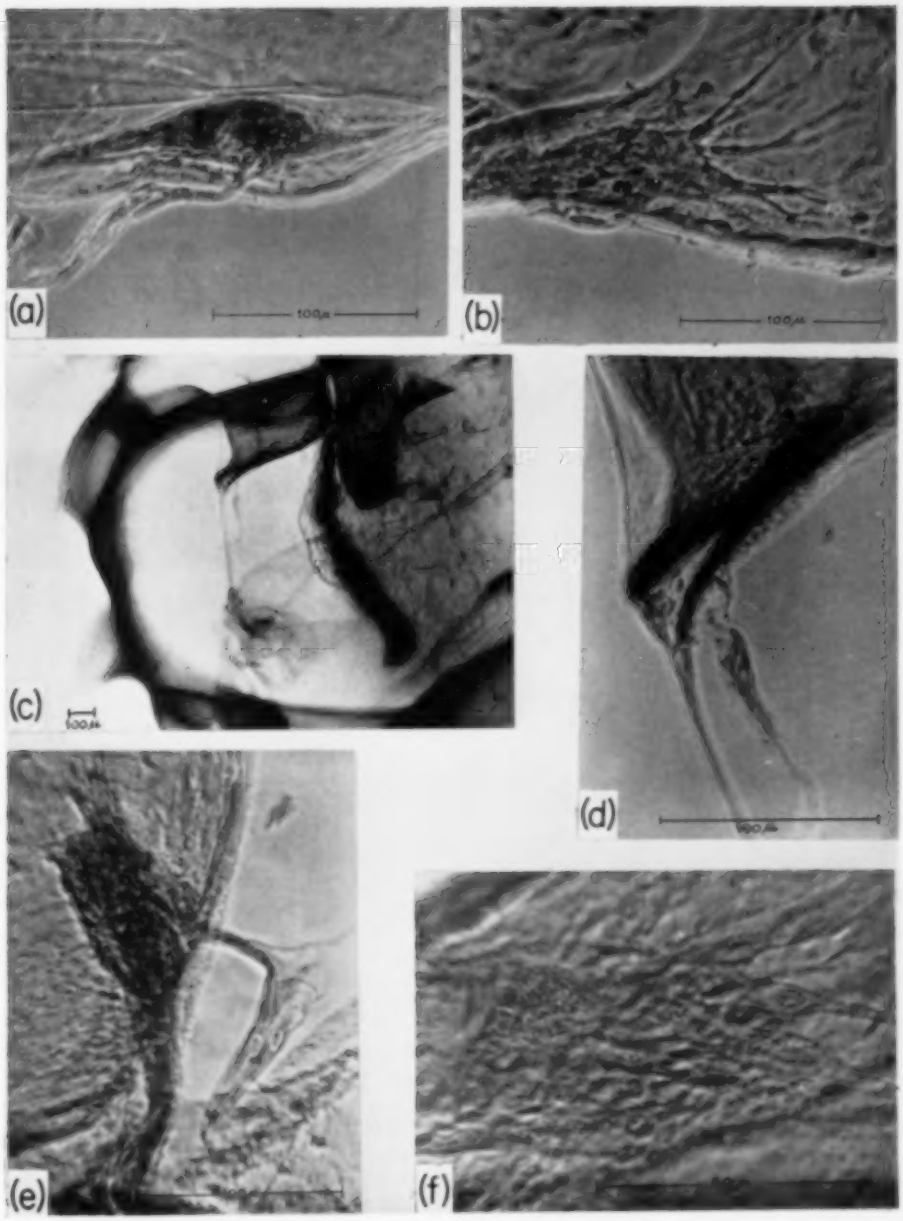


FIG. 3. (a) *Prodenia ornithogalli* Gn., ♂. Fixed methylene blue preparation showing portion of *Bügel* with B cell (centre) and portion of nerve entering (below) from scoloparium and turning leftward to accompany axon of B cell. The cell in this species appears fusiform, with a single, tapering tentacular process (right). This and all other photographs were taken using phase contrast microscopy. (b) *Peridroma margaritosa* Haw., ♀. Fixed methylene blue preparation showing branched tentacular processes of B cell. (c) *Agrotis ypsilon* Rott., ♀. Flemming-Hermann preparation, unstained, showing portion of right tympanum. Cf. drawing, Fig. 1. The B cell appears as a dark streak obliquely crossing the ventral margin of the *Bügel*. The faint streak extending thence ventrally is the tympanic nerve. (d) Detail of (c) above, showing nuclei of tracheal epithelial cells partly obscuring the B cell. Fibres from the scoloparium enter from below, right. (e) *Prodenia eridania* Cramer, ♂. Flemming-Hermann preparation, unstained, showing B cell (left centre), nuclei of the two acoustic sensory cells (below right centre) and fibres leading from them to *Bügel*. Faint streak extending to extreme right from bend of acoustic fibres is the ligament. Cf. drawing, Fig. 1. (f) *Pseudaletia unipuncta* Haw., ♂. Formalin injection followed by Flemming-Hermann fixation. Portion of *Bügel* under oil immersion, showing nucleated tentacular processes of B cell.

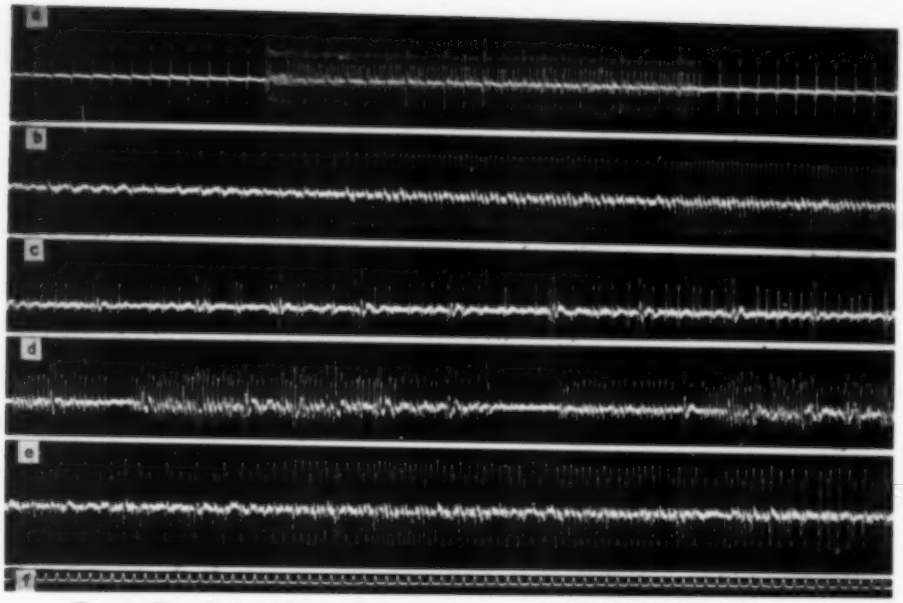


FIG. 4. Electrical activity in the tympanic nerve of noctuid moths. (a), *Catocala* sp. Tympanic organ intact. Brief acoustic response to a pure tone of 26 kc/sec, 0·1 V. The frequency of the B fibre impulses is unaltered during the acoustic response. The larger spikes of the B fibre extend above the envelope created by the A fibre response. (b), The same specimen. No acoustic stimulation. The frequency of the B impulses is being altered by manipulation of the *Bügel* membrane through the opened air sac. (c), *Prodenia eridania*. Continuous exposure to a pure tone of 50 kc/sec. The partially adapted acoustic response is somewhat irregular while the B impulses recur with their usual regularity. (d), The same during periodic manipulation of the skeletal frame of the tympanum. The mechanical distortion causes changes in the frequency of A and B spikes that are in phase. (e), *Catocala* sp. Manipulation of the *Bügel* membrane with a fine needle during exposure to a continuous pure tone. The partially adapted acoustic response shows changes in frequency as in figure (b) above. (f), Time calibration for all records: 100 pulses/sec.

homologue (IIIN1b) of the tympanic nerve in three atympanous moths. Two of these were sphingids and one was the citheroniid *Anisota rubicunda* Fabr. In none was any afferent activity observed.

From the above evidence it seems reasonable to conclude that the B neurone is a characteristic feature of the tympanic organs of noctuid moths, and perhaps also of geometrids as well.

Physiology. The apparently spontaneous, continuous, and more or less regular discharge of impulses here attributed to the B neurone has been described in a previous publication (ROEDER and TREAT, 1957), although at that time the source of these impulses was not known. The frequency varies from 3 or 4/sec to about 300/sec in different preparations, but in any one preparation tends to remain constant for long periods. The rate most commonly observed is 10–20 impulses/sec (e.g. Fig. 4 (a), (c)). Rarely, an almost total absence of spontaneous discharge has been observed, and in these few instances (1 *Prodenia eridania* ♀, 3 *Pseudaletia unipuncta* ♀♀) the acoustic sensitivity was as high as in other preparations. High B frequencies also accompanying normal acoustic thresholds are seen occasionally. The frequency of the B impulses was never observed to change during acoustic stimulation. In Fig. 4 (a) the tympanic organ is exposed briefly to a pure tone. The sequence of B spikes continues without change in frequency during the discharge of one of the acoustic fibres. The records show no features which appear characteristic of individual species or of sex, nor are any diurnal rhythms suggested. When simultaneous records are taken from both right and left tympanic nerves while these are still connected to the pterothoracic ganglion, it is apparent that the B impulses from the two sides are not synchronized, and except for general similarity in discharge rate, are quite independent of each other. The impulses are not affected by decapitation. They occur in preparations having all central connexions intact.

That the B cell is actually the source of these impulses was shown by the experiment of perforating first the tympanic membrane, then the *Bügel* sheath, and finally the cell itself with a KCl-filled glass micro-electrode. In this experiment, the head and legs of the moth were first removed, and an indifferent electrode was inserted into the abdomen. The moth was held by its upraised wings. When the electrode reached the B cell, trains of non-acoustic impulses were recorded, varying in periodicity when the electrode position was shifted slightly with the micro-manipulator. With the electrode so placed, no activity could be detected in the acoustic fibres, although the scoloparium was still intact. Similar results were obtained when the cell was approached from within the opened tympanic air sac in a hemisected specimen.

Soon after the non-acoustic impulses were first noted, it was discovered (ROEDER and TREAT, 1957) that their frequency could be greatly and reversibly altered by deformation of the metathorax along the longitudinal axis (Fig. 4 (d)), or by any manipulation that would change the tension in the sleeve of tracheal epithelium ensheathing the tympanic nerve as it leaves the region of the *Bügel* (Fig. 4 (b), (e)). Stretching the sheath increases the impulse frequency, while

VOL. 3 1959

relaxing the tension reverses this effect. The B cell does not adapt to the altered tension, but maintains the altered rate as long as the amount of stretch remains constant.

In view of its sensitivity to stretching, and of its close anatomical relation to the auditory organ, it was tempting to suppose that the B cell might in some way affect acoustic sensitivity, perhaps in a manner analogous to that of the tensor tympani in the mammalian ear. With this possibility in mind we sought to influence the threshold for the acoustic response by various manipulations affecting the B cell. The results were equivocal. When changes in B frequency were brought about indirectly by deformation of the thoracic exoskeleton, some preparations showed parallel and in-phase changes in the acoustic threshold. If the skeletal distortion was initiated when the acoustic units had reached a state of partial adaptation to a continuous tone, the A spikes could be made to recur at frequencies above and below their steady, adapted discharge rate (Fig. 4 (d)), while the B spikes underwent frequency changes in phase with those of the A spikes. On the other hand, when the nerve was stretched directly by movements of the electrode with the micro-manipulator, no change could be detected in the acoustic sensitivity, even when the discharge rate of the B cell was greatly increased (Fig. 4 (e)). Since reflex pathways in these preparations had been interrupted by the sectioning of the tympanic nerve, it is clear that whatever changes were observed were the direct results of the experimental manipulations.

We next tried to influence the discharge of the B cell by acoustic stimulation with clicks and pulsed sounds of varying intensity and periodicity. This gave only negative results. The repetition frequency of the clicks (and of the resulting volleys in the acoustic fibres) was adjusted to approximately that of the spontaneously active B cell. This gave no evidence of coupling or of any interaction between the acoustic and non-acoustic units.

If acoustic sensitivity is directly affected by the B neurone, it is in a manner too subtle for our methods of detection, although the possibility of an indirect, reflex effect has not been excluded. It has been pointed out above that the non-acoustic discharge is not affected by the destruction of the scoloparium, and that the acoustic response may survive the experimental inactivation of the B neurone, though with a reduced sensitivity which may reflect incidental damage to the acoustic nerve fibres.

In seeking a clue to the function of the B neurone, we have tried a variety of experimental devices. These include a vibrating tilt table adjustable to various angles to test for gravitational and accelerational sensitivity, and a variable pressure chamber to test for barostatic function. No significant response to any of these influences was detected. Chilling, heating, and drying, exposure to light, to carbon dioxide, and to nitrogen caused only such changes as would be expected in any excitable cell. Preparations in which it was possible to manipulate the wing stumps failed to show significant changes in B impulse frequency in response to this procedure, but since the forewings and the entire mesothoracic dorsum with its associated flight muscles had been removed from such preparations it is impossible to draw any conclusions from them as to what may take place during

flight. An electronic strain gauge applied to the thorax of moths during stimulated, stationary flight indicated an amount of thoracic displacement or deformation comparable to that required for changes of B frequency, though it is not known whether these distortions are in directions such as to produce a stretching of the *Bügel* sheath. Indeed, the structure of the tympanic organ seems adapted to the prevention rather than to the promotion of flight-induced distortions (TREAT, 1959).

Akinesis or 'death-feigning' often follows rough handling in which temporary skeletal deformation might occur. Yet extensive damage to both scoloparium and *Bügel* does not in our experience regularly prevent the akinetic response, nor does it give rise to any other clear-cut behavioural deficiencies except for failure of the response to sound. Posture, alighting, and righting reactions are not conspicuously affected in tympanotomized moths, though for some hours flight may be less ready and less vigorous in such insects than in controls. Mating behaviour has not been studied following tympanotomy, but neither feeding nor egg laying is disturbed in any obvious way. Records of B cell impulses in late pupae and 'pharate adults' of *Prodenia eridania* show that the cell is at least potentially active at a stage when the tympanic air sac is still filled with liquid, and when flight, of course, is impossible.

VOL. 3 1959

Discussion. Both its demonstrated sensitivity to stretch and its resemblance to known stretch receptors in other arthropods suggest that the B neurone is some type of proprioceptor. Its intimate relation to the auditory organ suggests a function related to that of hearing. Yet no physiological movement of a sort that would surely supply a stretch stimulus to the cell is known in the intact insect, and no evidence of relation to auditory function has been found. While we have no information regarding the pathways of B cell impulses within the central nervous system, it is possible that they might contribute to the maintenance or suppression of a central excitatory state, perhaps concerned with variations in responsiveness to acoustic or other stimuli.

If, as is commonly supposed, the tympanic auditory organ represents the evolutionary adaptation of a primitively proprioceptive structure to an acoustic function, the intimate structural relation of the B cell to the tympanic organ might find a rational explanation even in the absence of a physiological interaction. It is even conceivable that the B cell might represent the relatively unmodified portion of the same ancestral structure which has also given rise to the scoloparium. A comparative study of the tetrascolopophorous abdominal tympanic organs of the geometrids and some other lepidopterous families might shed light upon this possibility.

REFERENCES

EGGERS F. (1919) Das thoracale bitympanale Organ einer Gruppe der Lepidoptera Heterocera. *Zool. Jb.* (*Anat.*) **41**, 273–376.

FINLAYSON L. H. and LOWENSTEIN O. (1955) A proprioceptor in the body musculature of Lepidoptera. *Nature, Lond.* **176**, 1031.

FINLAYSON L. H. and LOWENSTEIN, O. (1958) The structure and function of abdominal stretch receptors in insects. *Proc. roy. Soc.* (B) **148**, 433–449.

HASKELL P. T. and BELTON P. (1956) Electrical responses of certain lepidopterous tympanal organs. *Nature, Lond.* **177**, 139–140.

KENNEL J. VON and EGGERS F. (1933) Die abdominalen Tympanalorgane der Lepidopteren. *Zool. Jb.* (*Anat.*) **57**, 1–104.

ROEDER K. D. and TREAT A. E. (1957) Ultrasonic reception by the tympanic organ of noctuid moths. *J. exp. Zool.* **134**, 127–158.

TREAT A. E. (1959) The metathoracic musculature of *Crymodes devastator* (Brace) (Noctuidae) with special reference to the tympanic organ. In: *Studies in invertebrate morphology*. Smithsonian Miscellaneous Collections **137**: 365–377, with 16 plates.

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 271 *to* 292. *Pergamon Press Ltd., London. Printed in Great Britain*

INSECT ACTOMYOSIN
A FLOW BIREFRINGENCE STUDY*

K. MARUYAMA

Biological Institute, College of General Education, University of Tokyo, Meguro, Tokyo, Japan

(*Received* 11 *December* 1958)

Abstract—Flow birefringence properties of myosin B (natural actomyosin), myosin, F-actin, and synthetic actomyosin from honeybee thoracic muscle were investigated in detail with special reference to the interaction with adenosine triphosphate (ATP). The flow birefringence of insect actomyosin was very strong from the comparative biophysical viewpoint. The outstanding aspect of insect actomyosin is that the flow birefringence properties are not so sensitive to the action of ATP as seen in actomyosins from other animals. It is concluded that the interaction between myosin and actin exists to some extent in the presence of ATP. Based on the above-mentioned results, the characteristics of insect actomyosin are discussed in connexion with its physiological function.

VOL.
3
1959

INTRODUCTION

RECENT physicochemical studies on muscle suggest that the ATP-actomyosin system plays a fundamental role in muscular function from a comparative viewpoint (cf. SZENT-GYÖRGYI, 1951; WEBER and PORTZEHL, 1952; MARUYAMA and TONOMURA, 1957). Although insect flight muscle has a highly specialized function and structure (cf. HODGE, 1955; PHILPOTT and SZENT-GYÖRGYI, 1955), no marked difference has been observed between the physicochemical properties of insect actomyosin and actomyosins of animals belonging to different phyla (GILMOUR and CALABY, 1953; MARUYAMA, 1954a, b, 1957b, 1958b). However, in a preliminary experiment it was found that the amount of flow birefringence of insect actomyosin was much larger than that of vertebrates and was relatively little affected by ATP (MARUYAMA, 1957b).

Since the classical work of MURALT and EDSALL (1930a, b), it has been well known that the actomyosin solution is strongly birefringent and ATP affects profoundly its birefringence properties (DAINTY *et al.*, 1944; BINKLEY, 1948). Recently we analysed the molecular mechanism of the interaction of rabbit myosin B with ATP, using flow birefringence (DRF) technique (NODA and MARUYAMA, 1958a, b).

Here a detailed work on the DRF properties of myosin B (natural actomyosin), myosin, F-actin, and synthetic actomyosin from honeybee thoracic muscle will be presented with special reference to the ATP effect on these proteins.

* Biochemical Approach to Muscular Function in Insects. III. II in this series, cf. MARUYAMA (1957b).

MATERIAL AND METHODS

Material

In most cases, actively flying adult workers of the honeybee, *Apis mellifera*, were used as material. In a few cases, developing pupae and just-emerged adults were taken from the beehive. Details in handling the material were described in previous papers (MARUYAMA, 1957a, b ; 1958b). For comparison, tail muscle of the crayfish (cf. MARUYAMA, 1958a) and the striated portion of adductors of the clam (cf. MARUYAMA, 1957c) were used.

Preparation of proteins

Natural actomyosin or myosin B was prepared as described before (MARUYAMA, 1957b). The protein solution, finally dissolved in 0·6 M KCl at pH 7 and clarified for 30 min at 12,000 G, will be referred to as 'original sample' in the text. Usually the original sample was suitably diluted and clarified for 1 hr at 24,000 G. Unless otherwise stated, this 'clarified sample' was used for measurements.

Myosin A was extracted according to the procedures of SZENT-GYÖRGYI (1945 ; 1951) (KCl-myosin), of HASSELBACH and SCHNEIDER (1951) (PP-myosin) and of SZENT-GYÖRGYI (1951) (KI myosin). The purification procedure was the same as for myosin B except that precipitation was effected by lowering the ionic strength to 0·05. Pure myosin was prepared from myosin B by the method of A. WEBER (1956) (Weber's myosin). Myosin B was centrifuged for 3 hr at 10^5 G in a preparative ultracentrifuge in the presence of 0·6 M KCl, 1·5 mM $MgCl_2$, 0·02 M tris-hydroxymethyl-aminomethane (Tris) buffer, pH 7, and 5 mM ATP. After centrifuging, the upper four-fifths of the clear supernatant was carefully pipetted away and used as the myosin solution. The remainder was decanted into a test-tube and also tested for the DRF assay.

Actin was prepared according to the method of BARANY *et al.* (1957) without previous extraction of myosin. Muscle suspension was washed with 0·04 per cent $NaHCO_3$ and 0·01 M ethylenediaminetetraacetic acid (EDTA). Well-washed muscle residue was treated with acetone four times. The acetone powder was extracted with pure water and the extract was clarified by centrifuging for 1 hr at 24,000 G. The clear yellowish supernatant was used as G-actin solution. G-actin was transformed into F-actin by adding KCl and $MgCl_2$ to final concentrations of 0·1 M and 0·1 mM, respectively.

To make synthetic actomyosin, ATP-free myosin was combined with F-actin in the standard medium.

Flow birefringence tests

Measurements for flow birefringence were carried out in a flow birefringence apparatus (Rao Instrument Co. Model A-2) at 20–21°C, using a teflon cell. The procedure was exactly the same as that described by NODA and MARUYAMA (1958a). The test solution consisted of 0·6 M KCl, 0·02 M Tris buffer (pH 7·0), 1·5 mM $MgCl_2$, and a given protein of appropriate concentration.

Two variables were measured as a function of the velocity gradient (G) over a wide range, the extinction angle (χ) and the amount of flow birefringence (Δn).

The relative value of flow birefringence will be frequently given as an angle Δ° in this paper (MURALT and EDSALL, 1930b; cf. CERF and SCHERAGA, 1952). The absolute amount of the flow birefringence (Δn) is easily calculated by multiplying the Δ value by a factor of $3{\cdot}51 \times 10^{-8}$ in the present study.

Other tests

Viscosity of any protein solution prepared in this work was always tested by usual Ostwald viscometers of high velocity gradients (mean value, more than 1000 sec^{-1}) at 15°C. Viscosity of a highly diluted myosin B solution in the standard medium was determined in a capillary viscometer of mean velocity gradient of 100 sec^{-1} at 20°C.

ATPase tests were carried out as previously reported (MARUYAMA, 1957b).

VOL. 3 1959

Protein concentration

The protein concentration was determined by nitrogen analysis through micro-Kjeldahl procedure, using the factor of 6.

Reagents

ATP was purchased from the Sigma Chemical Co. as a crystalline sample. ATP was dissolved in water, neutralized to pH 7 with KOH and stored at −5°C. Just before use, the ATP solution was made up into 0·6 M KCl solution. Usually 1·0 ml of the ATP solution (10 mM) was added to 9·0 ml of the experimental solution at 20°C and 1·0 ml of 0·6 M KCl alone was put into the control solution. Tris was the product of the British Drug Houses Co.; Pyrophosphate (PP) was twice recrystallized.

RESULTS

MYOSIN B

Molecular size

The so-called myosin B or natural actomyosin extracted for 3 hr with Weber-Edsall solution and purified three times by the usual dilution-precipitation procedure showed a strong flow birefringence at a very low velocity gradient. It was observed that the relationship between χ and G at very low velocity gradients depends upon the protein concentration for concentrations as low as 0·05 per cent. Usually the protein concentration around 0·03 per cent was used for measurements. It is to be noted that no dilution effect, i.e. dissociation upon dilution, occurred even if placed overnight at 0°C in such a low protein concentration (cf. JOLY *et al.*, 1955; HIPPEL *et al.*, 1958).

In Fig. 1 is presented a typical result of χ-G curve of insect myosin B at low G. We can obtain from this curve the rotary diffusion constant (θ) at

$G = 0$ by the following formula of PETERLIN AND STUART (cf. CERF and SCHERAGA, 1952).

$$\theta = -4{\cdot}78 / \left(\frac{d\chi}{dG}\right) \tag{1}$$

where χ is expressed in degrees (°). The rotary diffusion constant at $G = 0$ was about 0·4 sec^{-1} and upon addition of ATP it was slightly elevated to about 0·5 sec^{-1} (Table 1). The unclarified original sample showed somewhat smaller

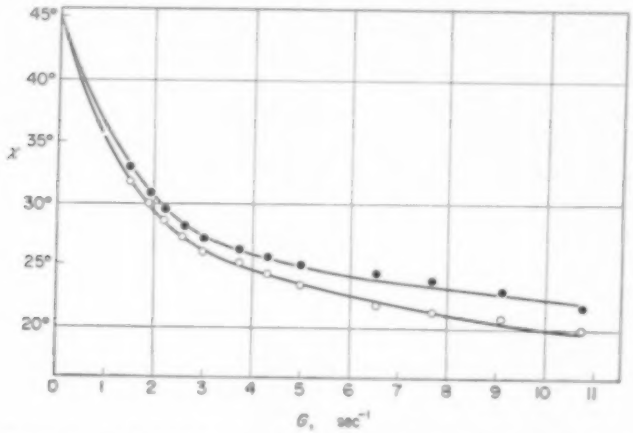


FIG. 1. Extinction angle (χ) of insect myosin B at low velocity gradients (G). 0·029 per cent; ○——○ : control; ●——● : with ATP (1mM).

TABLE 1—ROTARY DIFFUSION CONSTANT (θ) AND EXTINCTION ANGLE (χ) OF INSECT MYOSIN B

Conditions: 0·6 M KCl, 0·02 M Tris buffer, and 1·5 mM $MgCl_2$. 1 mM ATP, when added. pH 7·0; 20°C

Preparation No.	−ATP			+ATP		
	θ	χ		θ	χ	
	$G = 0$* (sec^{-1})	100 (°)	1200 (°)	$G = 0$ (sec^{-1})	100 (°)	1200 (°)
1	0·4	9·8	5·6	0·5	10·8	7·2
2	0·5	11·3	6·2	0·6	13·4	8·0
3	0·3	10·3	5·2	0·4	11·8	7·3
4	0·4	10·0	5·2	0·5	12·3	7·3
5	0·6	10·8	6·0	0·7	12·2	8·0
Average	0·44	10·4	5·6	0·54	12·1	7·6

* G is given as sec^{-1}.

θ values: 0·25 and 0·30 sec^{-1} with and without ATP.* However, in this case the χ-G curve was not reversible upon shear stress: after being subjected to G up to 2000 sec^{-1}, χ at low G was irreversibly increased, although the change was smaller in the presence of ATP. At high G, χ was greater than the value of the clarified sample (cf. Fig. 11). Possibly in the original sample a larger size of molecules may be broken by shear stress.

At high G, χ was also slightly, but definitely raised with ATP (5°–7° at G = 1200 sec^{-1}), as shown in Fig. 2. This increase in χ with ATP is not so pronounced as in rabbit myosin B (NODA and MARUYAMA, 1958a, b).

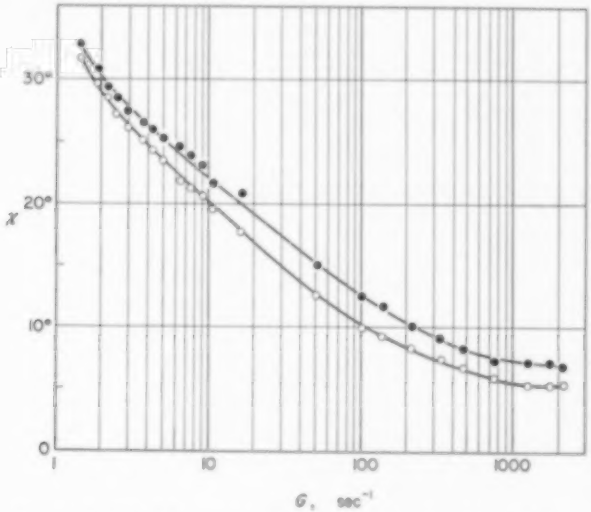


FIG. 2. Extinction angle (χ) of insect myosin B at a wide range of velocity gradients (G). 0·031 per cent; ○——○ : control; ●——● : with ATP.

The rotary diffusion constant (θ) is very sensitive to the length of molecules of a high degree of asymmetry. If we know θ and the axial ratio of any rigid prolate ellipsoid, we can easily calculate the molecular length by the following formula of PERRIN (cf. CERF and SCHERAGA, 1952).

$$a^3 = \frac{3kT}{16\pi\eta_0\theta}\left(-1+21n\frac{2a}{b}\right) \tag{2}$$

In this formula a and b are the semi-axes of the molecule, η_0 is the solvent viscosity, T is the absolute temperature, and k is the Boltzmann constant. It is a very difficult task to determine the axial ratio of a macromolecule of a very high degree of asymmetry such as actomyosin. As tentatively adopted by many workers in

* The anomalous behaviour of χ-G relationship observed in the unclarified sample of rabbit myosin B (NODA and MARUYAMA, 1958a) was not observed in the insect, although an aggregate formation was observed to take place with ATP, if temperature was raised from 0°C to 20°C after the addition of ATP.

this field (MEHL, 1940; TONOMURA and MATSUMIYA, 1958; NODA and MARUYAMA, 1958a), an axial ratio of 100 is used here. At given G, θ can be calculated from the table of EDSALL *et al.* (1951).

The rotary diffusion constants of insect actomyosin at various G are given in Table 2. They distributed from 0·6 to 12 sec^{-1} and 0·7 to 17 sec^{-1} with and without ATP, which correspond to molecular lengths of 3·3–1·2 μ, and 3·0–0·9 μ

TABLE 2—ROTARY DIFFUSION CONSTANT OF INSECT MYOSIN B AT VARIED VELOCITY GRADIENTS

Velocity gradient (sec^{-1})	Rotary diffusion constant (sec^{-1})		
	Control	ATP	PP
0	0·63	0·73	0·70
5	0·77	0·85	0·78
10	1·16	1·19	1·14
50	2·08	2·90	2·50
100	3·00	3·70	3·70
200	3·75	5·89	6·38
500	6·0	9·2	11·2
800	9·6	13·6	16·2
1200	12·6	19·2	23·5

Conditions as in Table 1. The concentration of ATP or PP was 1 mM, when added.

with and without ATP. This distribution is similar to rabbit actomyosin (3·0–1·0 μ) (NODA and MARUYAMA, 1958a; TONOMURA and MATSUMIYA, 1958).

Flow birefringence

Intensity of flow birefringence was also a little affected by ATP: it was only decreased by some 20–30 per cent up to 10 sec^{-1} and at more than 100 sec^{-1} no

TABLE 3—FLOW BIREFRINGENCE OF INSECT MYOSIN B

Preparation No.	Flow birefringence (Δn)*							
	−ATP				+ATP			
	G =5	10	100	1200	G =5	10	100	1200
1	101	157	272	369	81	117	257	363
2	95	134	220	311	77	103	182	297
3	79	115	217	335	64	96	195	341
4	104	139	242	342	75	106	209	339
5	87	133	213	320	65	104	202	337
Average	93	135	234	335	73	106	210	340

* Δn is given as 10^{-8} per 0·1 per cent protein solution under the conditions as in Table 1.

significant decrease was found (Fig. 3). In fact it was frequently observed that Δn somewhat increased with ATP at $G = 1200$ sec^{-1} (see Table 3 and Fig. 3). On the other hand, PP was more effective than ATP in lowering Δn of insect myosin B (Fig. 3).

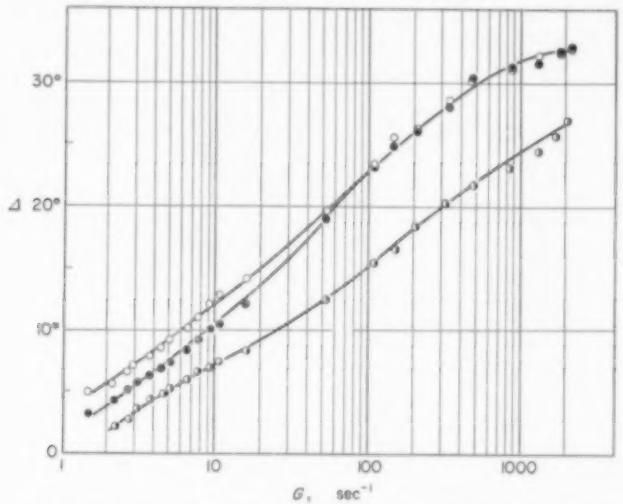


FIG. 3. Flow birefringence (Δn) of insect myosin B at a wide range of velocity gradients (G). 0·031 per cent; ○ : control; ● : 1 mM ATP; ◑ : 1 mM PP.

The effect of 0·5 M KI in addition to 0·1 M KCl was very remarkable on the DRF properties of insect myosin B. Whereas Δn of an original sample was 292×10^{-8} at $G = 1200$ sec^{-1}, it was lowered to 50 with KI (280 with ATP). The extinction angle was elevated from 6·7° to 25° (8·3° with ATP). The rotary diffusion constant in the presence of KI ranged from 100 to 1000 sec^{-1} at velocity gradients from 500 to 4000 sec^{-1}. Here again the KI effect was not so tremendous as in the rabbit (cf. NODA and MARUYAMA, 1958a).

MYOSIN A PROCEDURES

KCl-myosin

Short extraction of muscle with Weber-Edsall or Guba-Straub solution always yielded proteins of myosin B type in accordance with the results of GILMOUR and CALABY (1953) on locust muscle.

PP-myosin

HASSELBACH-SCHNEIDER'S procedure (1951), using PP, also yielded myosin B, the viscosity of which was greatly reduced with ATP, although the specific viscosity itself was considerably lower than that of 3 hr-myosin B (Table 4).

PP-myosin A showed similar flow birefringence properties to myosin B. Δn per unit protein concentration is comparable; θ at $G = 0$ is near 0·3 sec^{-1}. ATP somewhat increased θ. On the other hand, Δn was not appreciably lowered with ATP; one sample of three showed an appreciable increase in Δn at all G tested in the presence of ATP. The latter fact was never encountered in myosin B.

TABLE 4—VISCOSITY DATA OF MYOSIN A AND B FROM HONEYBEE THORACIC MUSCLE

Conditions: 0·6 M KCl; pH 7·0; 15°C

Preparation		Z	Z_{ATP}	ATP sensitivity
KI-myosin A		0·138	0·136	per cent 2
PP-myosin A,	1	0·243	0·121	100
	2	0·232	0·093	150
Myosin B,	1	0·420	0·180	130
	2	0·370	0·170	120
	3	0·380	0·180	110
	4	0·410	0·175	130
	5	0·390	0·175	120
	Average	0·394	0·176	124

KI-myosin

It was found that the only way to extract a solution of myosin directly from honeybee thoracic muscle was to use 0·6 M KI as the extracting medium (A. G.

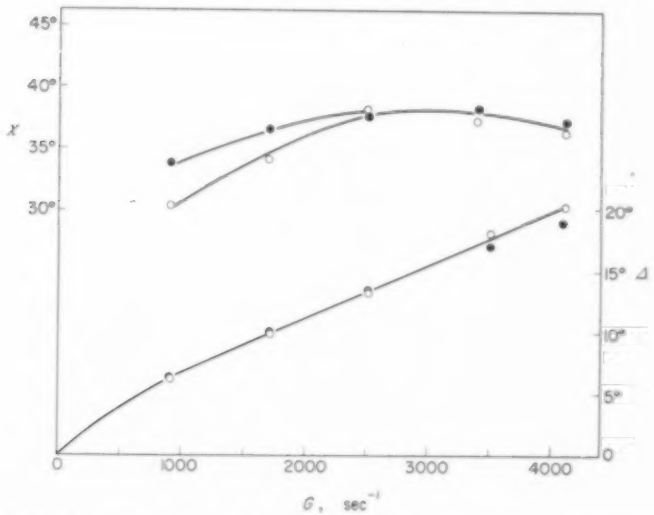


FIG. 4. Flow birefringence properties of insect KI-myosin. 0·1 per cent; ○——○ : control; ●——● : with ATP.

SZENT-GYÖRGYI, 1951). The KI-myosin showed no detectable viscosity change on addition of ATP. Table 4 summarizes the viscosity data of several kinds of myosin A and myosin B preparations from honeybee muscle. Here the ATP sensitivity of WEBER and PORTZEHL (1952) was adopted:

$$\text{ATP sensitivity} = \left(\frac{Z - {}^{Z}_{ATP}}{{}^{Z}_{ATP}}\right) \times 100 \qquad (3)$$

Z, viscosity number = $1n\ \eta\ rel/C$; C is the protein concentration given as g/l.; ${}^{Z}ATP$, viscosity number in the presence of ATP.

The flow birefringence properties of KI-myosin are quite different from those of PP-myosin or myosin B. As is seen in Fig. 4, Δn per unit protein concentration was very low at low G and increased proportionally to G up to 3000 sec^{-1}. ATP gave negligible effect on the Δn-G relationship. Furthermore, χ-G relationship was anomalous; at lower G, χ showed a low value and increased significantly in increasing G up to 2500 sec^{-1}. This fact is understood by assuming that a minute amount of larger particles, possibly actomyosin, contaminated the preparation. This was supported by the fact that ATP appreciably raised χ values at G lower than 2000 sec^{-1} (Fig. 4).

Weber's myosin

Pure myosin was separated from myosin B according to the method of A. WEBER (1956). A 0·3 per cent solution of myosin B was centrifuged for 3 hr at

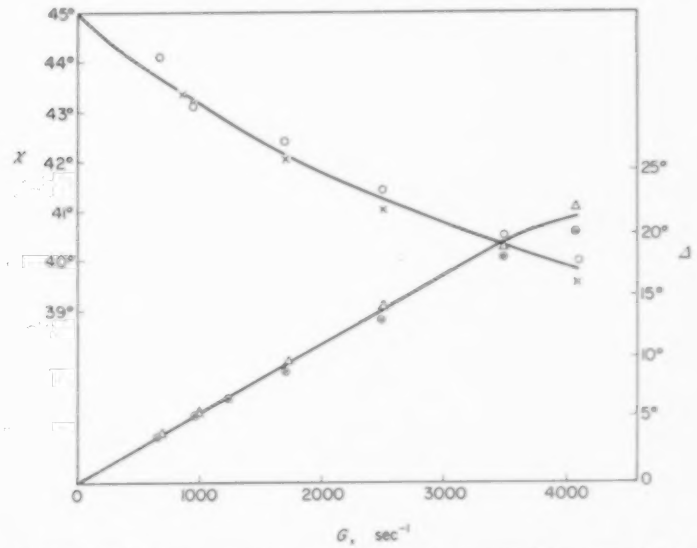


FIG. 5. Flow birefringence properties of insect myosin separated from myosin B. 0·1 per cent; extinction angle (χ) : ○, × ; flow birefringence (Δ) : ○, △. Different symbols show different preparations.

100,000 G in the presence of ATP. The water-clear solution of the upper supernate contained about 0·1 per cent of protein. The χ-G relationship was relatively homogeneous, giving a θ value of 2300 sec^{-1} at $G = 0$ (Fig. 5). The Δn-G curve was similar to that of KI-myosin, giving a Δn value of 25×10^{-8} at $G = 1200$ sec^{-1} per 0·1 per cent solution, which is in accord with the value obtained with rabbit myosin within the experimental error (NODA and MARUYAMA, 1958a). The length of insect myosin is rather uniform: 2000 Å–1700 Å was obtained (Table 5).

TABLE 5—ROTARY DIFFUSION CONSTANT (θ) AND APPARENT LENGTH (2a) OF INSECT WEBER'S MYOSIN AT VARIOUS VELOCITY GRADIENTS (G)

G (sec^{-1})	θ (sec^{-1})	2a (Å)
0	2300	2000
1000	2500	1960
2000	3100	1810
3000	3300	1780
4000	3600	1720

The length of rabbit myosin was 2200 Å at $G = 0$ (NODA and MARUYAMA, 1958a; JOLY *et al.*, 1955).

This fraction was dialysed to 0·6 M KCl, pH 7, for 48 hr, changing frequently the surrounding medium. After the added Mg and ATP were almost completely removed, the protein was tested for ATPase activity. The results for the ATPase assay are summarized in Table 6, which shows typical ATPase properties of

TABLE 6—ATPASE ACTIVITY OF INSECT WEBER'S MYOSIN

Conditions: 3 μM ATP, 0·6 M KCl, 0·02 M Tris buffer and 0·4 mg protein. Total volume, 1·5 ml. Incubated for 5 min at pH 7·0 and 20°C

Addition	μg P liberated
Control	2·0
1·5 mM $MgCl_2$	1·8
1·5 mM $MgCl_2$ + 1·5 mM $CaCl_2$	1·4
1·5 mM $CaCl_2$	15·5
3 mM EDTA	50·0

myosin. It should be noted that insect myosin splits only the terminal P group from ATP. The activity level itself was considerably higher than that of the corresponding myosin B under the similar conditions (cf. MARUYAMA, 1957b). The formation of actin complex gives final evidence that the fraction separated from myosin B is actually myosin (see over).

ACTIN

F-actin in myosin B

The presence of actin in rabbit myosin B was suggested by WEBER (1956) and later directly shown by BARANY *et al.* (1957) and GERGELY and MARTONOSI (1958). In the present study, a trial to extract actin from the precipitate formed upon the high speed centrifugation in the separation of myosin from myosin B was unsuccessful. However, there was obtained some evidence to suggest that actin was present in myosin B. The lower one-fifth of the supernate was tested for DRF measurements: a highly anomalous χ-G relationship was observed as seen in Fig. 6. This shows the presence of longer particles in addition to myosin. When

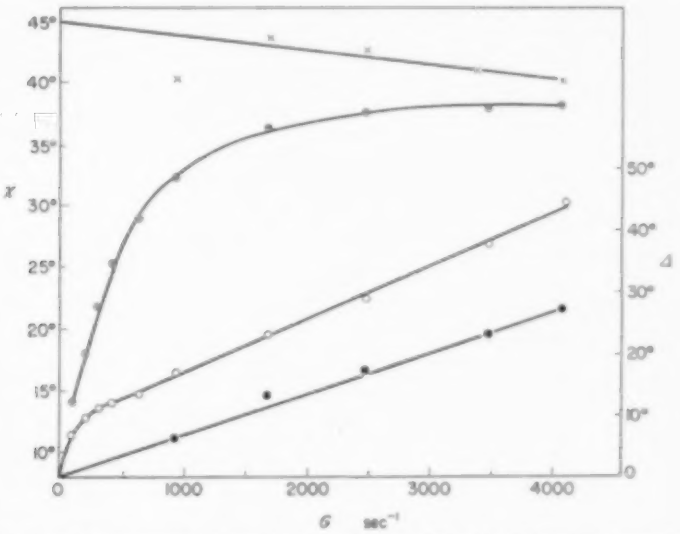


FIG. 6. Flow birefringence properties of a mixture of F-actin and myosin separated from insect myosin B. 0·23 per cent. Extinction angle (χ) : ○ (control); × (with 0·6 M KI); flow birefringence (Δ) : ○ (control); ● (with 0·6 M KI).

solid KI was added, the anomalous behaviour in the χ-G curve disappeared and furthermore the Δn-G curve turned into a myosin type. In the actin, prepared by conventional procedures, KI depolymerized F-actin to G-actin so that F-actin might be responsible for the anomalous behaviour.

G- and F-actin

Addition of 0·1 M KCl and 10^{-4} M Mg^{++} transformed insect G-actin into F-actin, the process of which was followed by viscometry. A sample, which originally showed the relative viscosity of 1·02 became viscous, the viscosity increasing to 1·62 upon G-F transformation within 30 min at 20°C after the addition of KCl and $MgCl_2$. The G-actin solution was completely non-birefringent and F-actin solution showed a strong birefringence. It is a curious fact that the

viscosity of F-actin was frequently low, but exhibited a strong flow birefringence.

The flow birefringence properties of F-actin greatly depend upon the protein concentration. It is a very difficult problem to make a proper characterization of F-actin by DRF analysis. Even in very dilute protein solutions such as 0·02 and 0·03 per cent, the concentration-change was observed in χ-G relationships, especially at low G: θ at $G = 0$ was 0·03, 0·04 and 0·08 sec^{-1} for the solutions of 0·042, 0·033 and 0·024 per cent. The results for 0·024 per cent solution are illustrated in Fig. 7. In that concentration, the χ-G relationship was reversible

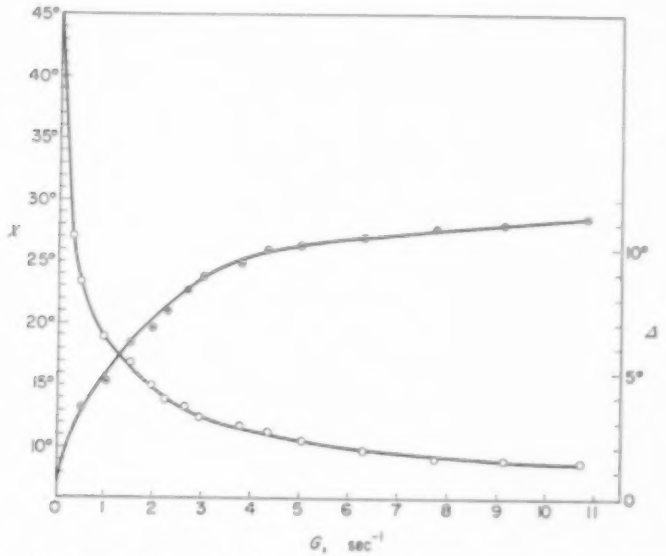


FIG. 7. Flow birefringence properties of insect F-actin. 0·024 per cent. Extinction angle (χ) : ○; flow birefringence (Δ) : ○.

up to $G = 20$ sec^{-1}. Once subjected to a high G, namely 1000 sec^{-1}, the strong flow birefringence of the solution did not disappear for a considerable time after the shear stress was removed. This behaviour more markedly increased in denser protein concentration. On the other hand, in very dilute solutions χ was irreversibly increased at high G.

The amount of Δn of F-actin was low in very dilute protein concentrations and it was very high in the dense ones. Besides, the Δn value varied widely at the same protein concentration and velocity gradient from sample to sample. These phenomena may be due to the formation and destruction of a complicated network of F-actin molecules, as shown in rabbit actin by OOSAWA and his collaborators (ASAKURA *et al.*, 1958). ATP did not give any effect on the DRF properties of F-actin.

SYNTHETIC ACTOMYOSIN

The F-actin solution and myosin fraction prepared from myosin B were mixed in several proportions and placed for at least 2 hr at about 20°C. The mixture formed actomyosin complex, showing response to ATP or PP. A typical result with the synthetic actomyosin is indicated in Figs. 8 and 9. The distinctive

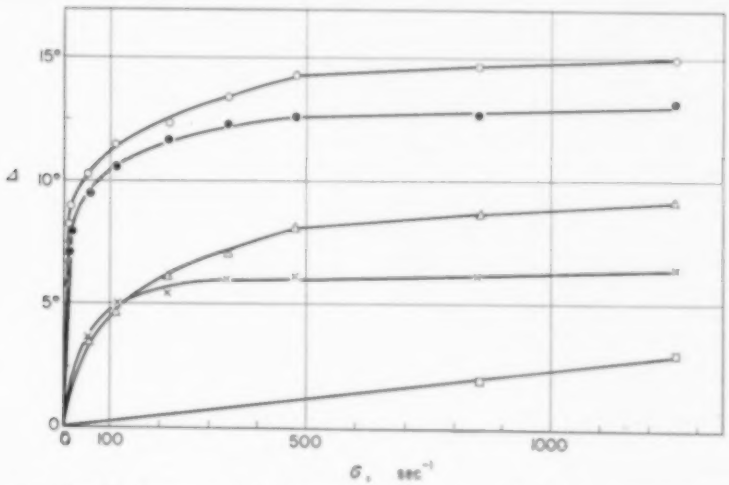


FIG. 8. Flow birefringence (Δ) of insect synthetic actomyosin and its components at high velocity gradients (G). ○ : 0·05 per cent actomyosin; ● : 0·05 per cent + ATP; △ : 0·05 per cent + PP; × : 0·025 per cent F-actin; □ : 0·025 per cent myosin.

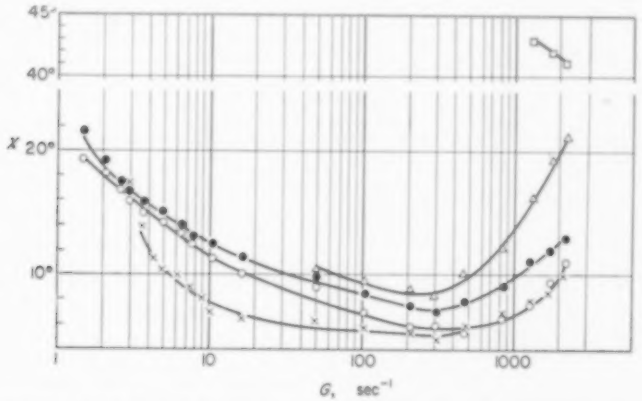


FIG. 9. Extinction angle (χ) of insect synthetic actomyosin and its components at a wide range of velocity gradients (G). Symbols as in Fig. 8.

aspects of the synthetic actomyosin are as follows: (1) ATP was more effective than on the natural actomyosin, i.e. the decrease in Δn was observed in all G ranges tested and χ was considerably elevated. (2) PP appeared to dissociate the

actomyosin into F-actin and myosin completely (see also Table 10). The latter fact with respect to Δn is clearly seen in Fig. 8. These results suggest that the actomyosin complex reconstituted *in vitro* was loose in the combination between myosin and F-actin. However, in the two kinds of preparations, where the myosin-actin ratio was 2 : 1 or 5 : 1, χ was considerably increased upon elevating G, especially at high G. This suggests that there existed free myosin or that myosin was set free from the complex by shear stress. In any case, ATP or PP gave a profound effect by causing a dissociation. It is of importance to note that Δn of the synthetic actomyosin was considerably smaller than that of the natural actomyosin: 105×10^{-8} (myosin–actin ratio,* 1 : 1), 74 (1 : 2) and 50 (1 : 5) per 0·1 per cent solution at $G = 1200$ sec^{-1}.

COMPARATIVE STUDIES

Comparison in different animals

It is highly significant that the flow birefringence of insect actomyosin is greatest in the actomyosins from various types of striated muscle so far investigated (Table 7). Although the rotary diffusion constants are of similar magni-

TABLE 7—A COMPARATIVE STUDY OF FLOW BIREFRINGENCE PROPERTIES OF MYOSIN B FROM DIFFERENT TYPES OF STRIATED MUSCLE

Animal	Type of muscle	ATP	θ $G = 0$ sec^{-1}	χ		Δn	
				$G = 200$ (°)	$G = 1200$ (°)	$G = 10$ (10^{-8})	$G = 1200$ (10^{-8})
Rabbit*	skeletal	−	0·7	10	8	47	164
		+	1·2	13	14	11	68
Honeybee	thoracic	−	0·4	8	5·6	135	335
		+	0·5	10	7·6	106	340
Crayfish	tail	−	1·8	10	11	38	115
		+	—	25	38	—	30
Clam	adductor	−	—	28	24	—	47
		+	—	38	36	—	25

* Data taken from NODA and MARUYAMA (1958a).

tudes, the birefringence of insect actomyosin is twice that of rabbit. The birefringence of crayfish or clam actomyosin is smaller than that of insect or rabbit actomyosin. One of the reasons why Δn of myosin B per unit protein concentration varies widely in different animals may be related to the extent of contamination by myosin, as suggested by the rise in χ values at high G as in the crayfish or clam (cf. MARUYAMA, 1959). It is rather striking that the ATP effect is the smallest in insect myosin B.

* The ratio was based on the protein concentration (gramme per litre). However, the concentration of the actin may be somewhat overestimated, because actin was not highly purified.

Changes during pupal and post-emergence development

As previously reported (MARUYAMA, 1957a, b), the ATP-actomyosin system in honeybee thoracic muscle changes a little during pupal and post-emergence development: in the mid-stage of pupal development there is a sudden increase

TABLE 8—FLOW BIREFRINGENCE PROPERTIES OF MYOSIN B FROM HONEYBEE MUSCLE DURING PUPAL DEVELOPMENT

Stage	ATP	θ	χ		Δn		
		$G = 0$ sec^{-1}	$G = 500$ (°)	1200 (°)	$G = 10$	100	1200
Pupa, mid stage	—	3·0	10·1	8·0	45	124	206
	+	4·8	14·0	11·7	32	85	182
Pupa, later stage	—	2·3	7·9	6·7	62	138	224
	+	—	10·5	9·4	40	97	182
Adult, just emerged	—	1·5	7·0	6·6	116	210	289
	+	1·7	9·5	8·4	85	192	282
Adult, active	—	0·5	6·6	5·9	135	250	325
	+	0·6	8·1	6·7	117	228	353

in the actomyosin content, and thereafter no marked changes were observed in the activity level of ATPase or in the physicochemical properties, including viscosity and sedimentation constants with and without ATP.

Unexpectedly, the flow birefringence properties were found to change in a characteristic way during metamorphosis. As summarized in Table 8, θ gradually

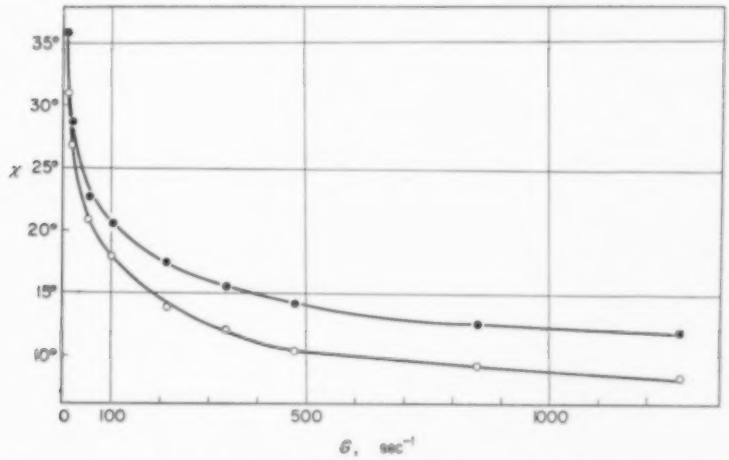


FIG. 10. Extinction angle (χ) of myosin B from honeybee mid-pupa at high velocity gradients (G). 0·05 per cent; ○——○ : control; ●——● : with ATP.

decreased, while Δn increased during pupal and post-emergence development. In addition, it should be emphasized that the effect of ATP was more pronounced in the earlier stages of development. In the mid-pupa, ATP considerably decreased Δn and increased χ in a wide range of G (Fig. 10).

DISCUSSION

THE INTERACTION WITH ATP

The most outstanding aspect of the interaction between insect myosin B in solution and ATP is the minor change in the DRF properties, although the viscosity, sedimentation pattern, and turbidity change greatly (MARUYAMA, 1957b), in contrast to crayfish myosin B (MARUYAMA, 1959).

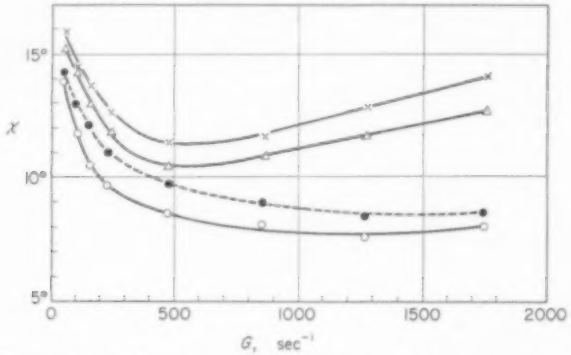


FIG. 11. Change in (χ–G) relationship in mixtures of insect myosin B and myosin. ○ : 0·02 per cent myosin B (original sample); ● : 0·02 per cent + ATP; △ : 0·02 per cent + ATP + 0·05 per cent myosin; × : + ATP + 0·07 per cent myosin.

TABLE 9—FLOW BIREFRINGENCE PROPERTIES OF MIXTURES OF INSECT MYOSIN B AND MYOSIN IN THE PRESENCE OF ATP

	χ*	Δ*
	(°)	(°)
0·02% myosin B	8·5	15·5
0·05% myosin	41·5	4·5
Myosin B + myosin	12·8	23·5
Theoretical value†	15·1	17·8

* $G = 1700$ sec^{-1}.
† Calculated from Sadron's formulae (4, 5).

Since the original work of SZENT-GYÖRGYI (1945), it has been generally accepted that ATP dissociates actomyosin solution into myosin and actin and this dissociation theory was confirmed by a flow birefringence study (NODA and MARUYAMA, 1958a, b).

Judged from the change in Δn and χ, the complete dissociation of insect actomyosin into its components does not occur with ATP, and only a partial dissociation takes place. We are led to assume that the interaction between myosin and actin still exists in the presence of ATP. Namely, the interaction between myosin and actin is rather strong in the insect. As indirect evidence, the change in χ and Δn upon the addition of pure myosin to actomyosin in the presence of ATP is presented in Fig. 11 and Table 9. If there is no molecular interaction between the added myosin and myosin B, we can calculate the theoretical values of χ and Δn from the formulae of SADRON (cf. CERF and SCHERAGA, 1952):

$$\tan 2\chi = \sum_i \delta_i \sin 2\psi_i / \sum_i \delta_i \cos 2\psi_i \tag{4}$$

$$(\Delta n)^2 = (\sum_i \delta_i \sin 2\psi_i)^2 + (\sum_i \delta_i \cos 2\psi_i)^2 \tag{5}$$

where the i species of particles which, if present alone, would give the observed values: ψ_i and δ_i of the extinction angle and birefringence, respectively, at the same velocity gradients.

VOL. 3 1959

As listed in Table 9, the actual values of χ and Δn of the combined solution were appreciably different from the theoretical ones: Δn of the former was larger than the latter; on the other hand, χ was smaller. This fact suggests that there was certainly some kind of interaction between myosin and actomyosin in the presence of ATP. Very possibly myosin interacts with the actin partner in the myosin B particle. In fact, in the similar experiments with rabbit actomyosin, the theoretical values agree well with the observed ones (NODA and MARUYAMA, 1958a).

The decrease in Δn at low G with ATP may be possibly due to partial dissociation of actomyosin and also to breaking up of some kind of network of actomyosin particles (cf. TONOMURA and MATSUMIYA, 1958). The recovery of decreased Δn at high G may be due to the orientation of particles, but the complete explanation remains to be elucidated. ATP caused no detectable effect on Δn of myosin or of actin alone, respectively. The Δn of myosin, actin, and actomyosin were all positive so that we can expect that myosin particles attach to actin filaments, the major axes being parallel.

The influence of PP on the DRF properties of insect actomyosin merits special attention. The PP effect was more remarkable than the ATP one: χ at high G markedly increased and Δn decreased in the whole range of G (Fig. 3 and Table 2). In this case, dissociation certainly appears to have taken place to a considerable extent. In short, PP was more effective on insect actomyosin than ATP in the presence of a sufficient amount of Mg^{++}, although in rabbit, the action of ATP and of PP are quite equal (NODA and MARUYAMA, 1958). The effects of ATP and PP are more clearly seen in the reconstituted actomyosin than in the natural actomyosin described above (Figs. 2, 8, and 9). In the presence of PP, the χ and Δn values are approximately equal to those calculated from Sadron's formulae (4, 5), as seen in Table 10.

However, there arises some serious doubt as to the partial dissociation hypothesis in the presence of ATP. It is concerned with the profound decrease in the viscosity of insect actomyosin as well as the change in the sedimentation pattern in the presence of ATP (MARUYAMA, 1957a, b).

TABLE 10—FLOW BIREFRINGENCE PROPERTIES OF INSECT SYNTHETIC ACTOMYOSIN AND ITS COMPONENTS IN COMPARISON WITH THE THEORETICAL DATA

	χ^* (°)	Δ^* (°)
0·025% myosin	2·8	2·9
0·025% actin	7·3	6·3
0·05% actomyosin	7·7	15·0
0·05% + ATP	10·9	13·0
0·05% + PP	16·0	8·0
Myosin + actin†	17·9	7·8
0·033% myosin	42·8	3·8
0·017% actin	7·3	4·3
0·05% actomyosin	14·7	10·5
0·05% + ATP	18·0	9·1
0·05% + PP	21·0	7·0
Myosin + actin†	23·4	6·6

* Data at $G = 1200\ sec^{-1}$.
† Calculated from Sadron's formulae (4, 5).

The viscosity data shown in Table 4 were obtained by using the conventional Ostwald viscometer at high G and the solution of relatively higher protein concentration was employed for tests, where molecular interaction plays a predominant

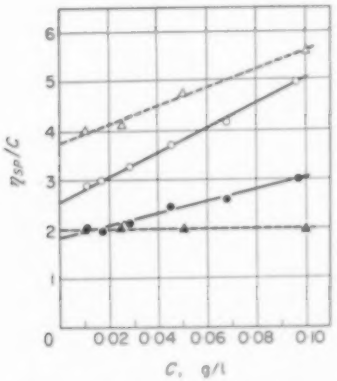


FIG. 12. Viscosity of insect and rabbit myosin B in dilute protein concentrations (C). Mean velocity gradient = $100\ sec^{-1}$; insect : ○ (control); ● (with ATP); rabbit : △ (control); ▲ (with ATP). The system consisted of 0·6 M KCl, 0·02 M Tris buffer and 1·5 mM $MgCl_2$ at pH 7·0 and 20°C ± 0·1°C.

role in the viscosity. Therefore the very dilute solution (from 0·01 per cent to 0·1 per cent) was tested for viscosimetry at mean G of 100 sec^{-1}. The results are shown in Fig. 12 in comparison with the rabbit. The decrease in viscosity with ATP was certainly found to a lesser extent in the insect than in the rabbit. Possibly the change in viscosity may be explained by the disappearance of the interaction between actomyosin molecules and of loosening of the actomyosin complex with ATP.

The separation of myosin from myosin B might be due to the shift of the following equilibrium to the right through the removal of actin by centrifugation:

$$AM \rightleftharpoons M + A$$

It is of great interest that the recovery of pure myosin in the supernatant was much smaller (30 per cent of the original concentration) in the insect than in the rabbit (60 per cent). It is not clear whether this is due to the larger amount of actin in the actomyosin or to the larger extent of the myosin-actin interaction. Both might be responsible.

VOL. 3 1959

COMPARATIVE VIEW

As described in the section 'Results', the Δn of insect actomyosin is very great in comparison with that of rabbit, crayfish, and clam myosin B. Since the orientation factors of crayfish and clams actomyosin are smaller, direct comparison at the same velocity gradient is not appropriate (CERF and SCHERAGA, 1952); in these protein solutions, pure myosin appears to have been a contaminant to a considerable extent. Nevertheless it is certain that the optical factor of insect myosin B is the greatest among the actomyosins tested. MEHL (1940) reported the DRF properties of (acto) myosins from several animals (snail, octopus, and sting ray), and the Δn of the preparations are much smaller than that of the insect under similar experimental conditions. In addition, ATP had much effect on the myosin B molecules from rabbit, crayfish, and clam muscles, but not on the insect myosin B.

It has been considered that, at least in the rabbit, myosin first dissolves into extracting medium, say Weber-Edsall solution, and then myosin is gradually transformed into actomyosin in combination with actin, although the precise situation remains obscure (NODA and MARUYAMA, 1958b). In insect muscle, this is not the case. As was shown in an elegant manner in locust muscle (GILMOUR and CALABY, 1953), and later confirmed by the present author's experiments (MARUYAMA, 1954a, 1957b, 1958b), actomyosin is directly solubilized from the beginning. This fact and the large birefringence suggest that the intact actomyosin filaments in the sarcomeres may be separated into the solution, retaining the structure *in situ* to some extent. The length of actomyosin approximates that of one sarcomere ($\sim 3\ \mu$) (PHILPOTT and SZENT-GYÖRGYI, 1955).

Hodge (1955) reported that birefringence of insect myofibrils decreases only slightly during contraction, which is the case in the flow birefringence of honeybee actomyosin sol with ATP. The relatively strong interaction between myosin

and actin might have somehow a close bearing upon the very quick contraction-relaxation cycle in insect flight muscle. With respect to this point, it is of importance that a greater degree of the interaction occurs upon the onset of the actual physiological function of the flight muscle in the honeybee life-span. The fine structure of myofibrils undergoes a marked development during pupal development of the honeybee (KOSHIHARA and MARUYAMA, 1958). Here, however, it should be pointed out that the respiratory enzyme systems in sarcosomes play a dominant role in the actual flight of the insect, as clearly shown in honeybee muscle (MARUYAMA and MORIWAKI, 1958). The special physicochemical characteristics of the ATP-utilizing system in myofibrils and the high efficiency of the ATP-supplying system in sarcosomes might make possible a very rapid contraction in insect flight muscle.

SUMMARY

Flow birefringence properties of myosin B or natural actomyosin, myosin, actin, and synthetic actomyosin from honeybee thoracic muscle were investigated with special reference to their interaction with ATP. Extraction of honeybee muscle with strong salt solution even in the presence of pyrophosphate always yielded myosin B, the viscosity of which was lowered with ATP. Direct extraction of myosin could be done, using 0·6 M KI solution as an extractant.

Flow birefringence properties of insect myosin B were only a little affected with ATP: the amount of flow birefringence was lowered with ATP only at low velocity gradients and rotary diffusion constant increased slightly, in a wide range of velocity gradients. Myosin was separated by ultracentrifuging in the presence of ATP, which reacted with F-actin to form actomyosin after ATP was dialysed away.

The molecular length of insect myosin B ranged from 3·3 to 1·1 μ and from 3·0 to 0·9 μ with and without ATP. Myosin was from 1700 to 2000 Å in length. The molecular dimension of F-actin was not clearly determined owing to its very complicated behaviour at a shear stress.

The comparative study of crayfish and clam myosin B as well as that of rabbits reveals a special behaviour of insect myosin B as regards the flow birefringence properties: the amount of flow birefringence per unit protein concentration was very high and the effect of ATP was relatively small, although PP was more effective than ATP.

It is tentatively suggested that the interaction between myosin and actin even in the presence of ATP exists to a considerable extent in insect myosin B. The present findings are also discussed in connexion with the physiological function of insect flight muscle.

Acknowledgements—I wish to express my sincere appreciation to Dr. H. NODA (Univ. Tokyo), where most of this work was carried out, for his helpful advice and stimulating discussion. I am deeply indebted to Drs. Y. TONOMURA (Hokkaido Univ.) and F. OOSAWA (Nagoya Univ.) for their kind criticism. My grateful thanks are also due to Professor J. ISHIDA (Univ. Tokyo) for his kind interest, and to Dr. D. R. KOMINZ (Univ. Tokyo; National Institutes of Health, Md.) for his help in the preparation of the manuscript.

REFERENCES

ASAKURA S., HOTTA K., IMAI N., OOI T., and OOSAWA F. (1958) G-F transformation of actin by divalent cations. *Conference on the chemistry of muscular contraction*, pp. 57–65. Tokyo.

BARANY M., BARANY K., and GUBA F. (1957) Preparation of actin without extraction of myosin. *Nature, Lond.* **179**, 818–819.

BINKLEY F. (1948) Birefringence of flow of preparations of myosin. *J. biol. Chem.* **174**, 385–387.

CERF R. and SCHERAGA H. A. (1952) Flow birefringence of macromolecules. *Chem. Rev.* **51**, 185–261.

DAINTY M., KLEINZELLER A., LAWRENCE A. S. C., MIALL M., NEEDHAM J., NEEDHAM D. M., and SHEN S. C. (1944) Studies on the anomalous viscosity and flow-birefringence of protein solutions. III. Changes in these properties of myosin solutions in relation to adenosinetriphosphate and muscular contraction. *J. gen. Physiol.* **27**, 355–399.

GERGELY J. and MARTONOSI A. (1958) The demonstration of actin in natural actomyosin (Myosin B). *Fed. Proc.* **17**, 227.

GILMOUR D. and CALABY J. H. (1953) Physical and enzymatic properties of actomyosins from the femoral and thoracic muscles in an insect. *Enzymologia* **16**, 23–33.

HASSELBACH W. and SCHNEIDER G. (1951) Der L-Myosin und Aktingehalt des Kaninchenmuskels. *Biochem. Z.* **321**, 462–475.

HIPPEL P. H. VON, GELLERT M. F., and MORALES M. F. (1958) Some physical and enzymatic phenomena in solutions in myosin B. *Conference on the chemistry of muscular contraction*, pp. 1–13. Tokyo.

HODGE A. J. (1955) Studies on the structure of muscle. III. Phase contrast and electron microscopy of dipteran flight muscle. *J. biophys. biochem. Cytol.* **1**, 361–380.

JOLY M., SCHAPIRA G., and DREYFUS J. C. (1955) Length of myosin particles in dilute solutions determined by flow birefringence. *Arch. Biochem. Biophys.* **59**, 165–180.

KOSHIHARA H. and MARUYAMA K. (1958) Changes in fine structure of honeybee thoracic muscle. *Sci. Pap. Coll. Gen. Educ., Univ. Tokyo* **8**, 213–216.

MARUYAMA K. (1954a) Studies on adenosinetriphosphatases of various insect muscles. *J. Fac. Sci., Tokyo Univ.* (4) **7**, 231–270.

MARUYAMA K. (1954b) The activity change of actomyosin-adenosinetriphosphatase during insect metamorphosis. *Biochim. biophys. Acta* **14**, 284–285.

MARUYAMA K. (1957a) Interaction between ATP and actomyosins from honeybee muscles during pupal development. *Z. vergl. Physiol.* **40**, 451–453.

MARUYAMA K. (1957b) A further study of insect actomyosin. *Sci. Pap. Coll. Gen. Educ., Univ. Tokyo* **7**, 213–241.

MARUYAMA K. (1957c) Contractile proteins from adductors of *Meretrix* and *Cristaria*. *Annot. zool. jap.* **30**, 63–66.

MARUYAMA K. (1958a) Contractile protein from crayfish tail muscle. *Biol. Bull., Woods Hole* **114**, 95–105.

MARUYAMA K. (1958b) Interaction of insect actomyosin with adenosine triphosphate. *J. cell. comp. Physiol.* **51**, 173–188.

MARUYAMA K. (1959) Flow birefringence studies of crayfish myosins. *Arch. Biochem. Biophys.* **82**, 422–430.

MARUYAMA K. and MORIWAKI K. (1958) Respiratory enzyme systems and muscular function in honeybee thoracic muscle. *Enzymologia* **19**, 211–219.

MARUYAMA K. and TONOMURA Y. (1957) Comparative biochemical studies of the ATP-myosin B system. *J. Res. Inst. Catalys. Hokkaido* **5**, 55–70.

MEHL W. J. (1940) Studies on the proteins of smooth muscle. II. The myosins of the octopus, snail, sea cucumber and sea anemone. *Biol. Bull., Woods Hole* **79**, 488–497.

VOL. 3 1959

MURALT A. L. VON and EDSALL J. T. (1930a) Studies in the physical chemistry of muscle globulin. III. The anisotropy of myosin and the angle of isocline. *J. biol. Chem.* **89**, 315–350.

MURALT A. L. VON and EDSALL J. T. (1930b) Studies in the physical chemistry of muscle globulin. IV. The anisotropy of myosin and double refraction of flow. *J. biol. Chem.* **89**, 351–386.

NODA H. and MARUYAMA K. (1958a) Interaction of myosin B with ATP. A flow birefringence study. *Biochim. biophys. Acta* **30**, 598–608.

NODA H. and MARUYAMA K. (1958b) Flow birefringence properties of various myosin B preparations. *Biochim. biophys. Acta* **32**, 263–264.

PHILPOTT D. E. and SZENT-GYÖRGYI A. (1955) Observations on the electron microscopic structure of insect muscle. *Biochim. biophys. Acta* **18**, 177–182.

SCHERAGA H., EDSALL J. T., and GADD J. O. (1951) Double refraction of flow: numerical evaluation of extinction angle and birefringence as a function of velocity gradient. *J. chem. Phys.* **19**, 1101–1108.

SZENT-GYÖRGYI A. (1945) Studies on muscle. *Acta Physiol. Scand.* 9, suppl. **25**, 1–128.

SZENT-GYÖRGYI A. (1951) *Chemistry of muscular contraction*, 2nd ed. Academic Press, New York.

SZENT-GYÖRGYI A. G. (1951) A new method for the preparation of actin. *J. biol. Chem.* **192**, 361–369.

TONOMURA Y. and MATSUMIYA H. (1958) Rotary diffusion of actomyosin. *Biochim. biophys. Acta* **27**, 311–318.

WEBER A. (1956) The ultracentrifugal separation of 1- myosin and actin in an actomyosin solution under the influence of ATP. *Biochim. biophys. Acta* **19**, 345–351.

WEBER H. H. and PORTZEHL H. (1952) Muscle contraction and fibrous proteins. *Advanc. Protein Chem.* **7**, 161–252.

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 293 *to* 305. *Pergamon Press Ltd., London. Printed in Great Britain*

VITAMIN DEFICIENCIES IN THE HOUSEFLY PRODUCED BY ANTIVITAMINS

ZWI H. LEVINSON*

Medical Research Laboratories, Army Medical Corps, Israel Defence Forces

ERNST D. BERGMANN

Scientific Department, Israel Ministry of Defence

(*Received* 18 *December* 1958)

VOL. 3 1959

Abstract—The effect of antivitamins on the housefly *Musca vicina* (Macq.) has been investigated using an artificial diet. Larval growth was found to be severely hampered by the presence of neopyrithiamine, desoxypyridoxine, 4-aminopteroylglutamic acid, pantothenol, pantoyltaurine, 3-acetylpyridine, α-picolinic acid, pyridine-3-sulphonic acid, benzimidazole, and *p*-aminoacetophenone, and to a lesser extent by avidin, *iso*-nicotinic acid, and *p*-aminobenzenesulphonamide. The rate of inhibition appeared to be proportional to the level of the antimetabolites in the diet. Thiamine and pyridoxine reversed completely the effect of neopyrithiamine and desoxypyridoxine, respectively, whereas folic, pantothenic, and nicotinic acids relieved only partly the action of the corresponding antivitamins. The growth inhibitory action of avidin was overcome by biotin addition or by heating. The toxic effect of benzimidazole or *p*-aminobenzenesulphonamide was not relieved by adenine or *p*-aminobenzoic acid.

On a synthetic diet consisting of casein, cholesterol, salts, nipagin M, thiamine, pyridoxine, pantothenic acid, nicotinic acid, folic acid, biotin, riboflavine, choline, and RNA, the larvae reached only $\frac{1}{3}$ of the weight attained on the artificial diet which contained yeast extract instead of the above water-soluble growth factors.

Oviposition is impeded when 4-aminopteroylglutamic acid, 3-acetylpyridine, desoxypyridoxine, neopyrithiamine, and pantothenol are added to the diet of the adult flies. Apart from growth inhibition, the vitamin deficiencies cause pathological features in larvae, pupae, and adults of the housefly.

INTRODUCTION

THE use of antimetabolites has become a standard procedure in the study of nutritional requirements of many types of organisms. Thus, pyrithiamine and neopyrithiamine were found to cause thiamine deficiency in mice and in bacteria and fungi requiring either thiamine or its pyrimidine or thiazole moieties (WOOLLEY and WHITE, 1943), whereas micro-organisms synthesizing thiamine were not affected by the antimetabolite (WOOLLEY, 1952). Benzimidazole is an antagonist of adenine and guanine in several micro-organisms but not in mammals (WOOLLEY, 1944). The chick embryo needs pyridoxine for its growth; the latter is inhibited by desoxypyridoxine, an antagonist for pyridoxine (OTT, 1946), and is restored by any of the three forms of vitamin B_6 (CRAVENS, 1952). In rats and chicks, the growth inhibitory action of 4-aminopteroylglutamic acid (aminopterin) is relieved only by relatively large amounts of folic acid, whereas in *Streptococcus faecalis* a

* Present address: Department of Zoology, University of Cambridge.

true competitive relationship was established between the vitamin and the antagonist (OLESON, HUTCHINGS, and SUBBAROW, 1948). Mammals can utilize pantothenol, as they are able to oxidize the latter to pantothenic acid, whereas in bacteria which cannot do so, pantothenol or pantoyltaurine inhibit competitively the utilization of pantothenic acid (SNELL and SHIVE, 1945). 3-Acetylpyridine causes niacin deficiency in mice, though not very efficiently (WOOLLEY and COLLYER, 1945), whereas the analogously constituted pyridine-3-sulphonic acid does not have this effect in mice (WOOLLEY and WHITE, 1943) and human beings (BEAN, SPIES, and BLANKENHORN, 1944). The sulphonic acid is inhibitory only for niacin-dependent bacteria (MCILWAIN, 1940) in the same manner as α-picolinic acid (MOELLER and BIRKOFER, 1942).

Very few studies have been devoted to the effect of antivitamins on insects. The influence of folic acid analogues on larvae of *Drosophila melanogaster* (GOLDSMITH, HARNLY, and TOBIAS, 1950; HINTON, 1952) and *Musca domestica* (MITLIN, KONECKY, and PIQUETT, 1954), and the competitive antagonism between β-butyrobetaine and carnitine in *Tenebrio molitor* (BHATTACHARYYA, FRIEDMANN, and FRAENKEL, 1954) have been studied. The substitution of several antivitamins for the corresponding vitamins in the diet of *Periplaneta americana* enabled SIEBURTH and MCLAREN (1953) to show that thiamine, nicotinic, pantothenic, and folic acids are essential to the larvae of this insect. SHYAMALA and BHAT (1958) have recently used 3-acetyl-pyridine and desoxypyridoxine to prove the niacin and pyridoxine requirement in the monophagous larva of *Bombyx mori*.

The experiments described in this paper were carried out in order to determine the effect of certain antivitamins on the larval growth of *Musca vicina* Macq. and their competitive relationship to the corresponding vitamins. Some experiments have also been devoted to adults of the same strain of houseflies.

MATERIALS AND METHODS

The experiments were performed with an artificial diet the composition of which is summarized in Table 1. This diet was prepared as follows: Cholesterol was dissolved in 20 ml of ether, the solution was poured into an Erlenmeyer flask (250 ml) containing the powdered filter paper and the solvent allowed to evaporate for 48 hr at 30°C, whereby an even distribution of the cholesterol was obtained. Casein, salts and nipagin M were then added and mixed thoroughly with the contents of the flask, which was stoppered subsequently by a plug of cotton wool, covered with tin foil and autoclaved for 20 min at 121°C. Five millilitres of a freshly prepared solution of 20 per cent (w/v) yeast extract, sterilized by filtration through a Seitz funnel, were added to the dry constituents of the diet.

The soluble antivitamins and vitamins were tested as such, whereas the insoluble ones were used in form of their salts as listed in Table 2; stock solutions of both types were prepared and sterilized subsequently by filtration. A filtered solution of fresh egg albumen (hen) was used to provide avidine. The stock solutions were diluted with sterile water to the desired concentrations. After bringing their water content to a volume of 17·5 ml (per flask), they were carefully added to

and well mixed with the other dietary constituents. This procedure was carried out just before the addition of newly hatched larvae to a series of flasks.

TABLE 1—COMPOSITION OF AN ARTIFICIAL DIET FOR HOUSEFLY LARVAE

Constituent	g	per cent
Casein, vitamin free (Nutritional Biochemicals Co.)	2·5	9·6
Yeast extract, anhydrous (Difco)*	1·0	3·8
Cholesterol	0·025	0·1
Salt mixture No. 2 (USP XIII)	0·016	0·06
Nipagin M	0·075	0·3
Water	22·5	86·0
Powdered filter paper (passing through sieve 10 mesh)	8·0	—

* A water soluble, lipid- and carbohydrate-free autolysate of *Saccharomyces cerevisiae*. A typical analysis of its major B-vitamins is recorded below :

Vitamin	mg/g dry weight
Thiamine	0·0032
Riboflavine	0·019
Pyridoxine	0·020
Nicotinic acid	0·279
Pantothenic acid	0·045
Biotin	0·0014

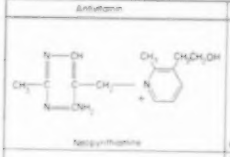
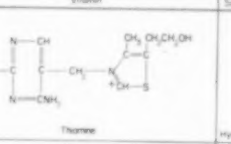
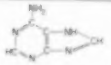
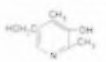
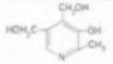
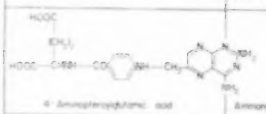
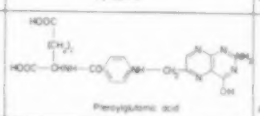
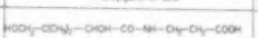
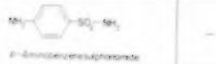
VOL. 3 1959

Newly laid eggs were obtained from a laboratory strain of *Musca vicina* grown in axenic culture with *Escherichia coli* on a mixture of 40 per cent (w/v) wheat bran and water.

The fly eggs were washed with dilute detergent solution, immersed for 30 min into a sterilizing solution containing formalin (5·0 per cent) and potassium hydroxide (1·0 per cent), and rinsed with sterile water. The larvae were then allowed to hatch into sterile water and were transferred immediately to the experimental diets. The method of evaluating larval growth and pupation has been described previously (LEVINSON and BERGMANN, 1957).

Due to similarity between the colours of diet and larvae and the small size of the latter, it was rather difficult to detect larvae which had grown only slightly or not at all. The following apparatus facilitated the detection of such larvae. An electric bulb (60 W) was fastened centrally inside a pyrex glass funnel (upper diameter 12 cm) which had been painted black on its external surface. The funnel was fixed vertically by a clamp and its upper opening covered by a white glass plate. An open petri dish (diameter 15 cm) was placed on it in such a way that the distance between the circumference of the dish and the funnel became approximately 1·5 cm (Fig. 1). The medium containing minute larvae was diluted with water and the resulting suspension poured in small amounts at a time into the petri dish. When the bulb was illuminated, the larvae fled immediately from the

TABLE 2

Antivitamin	Salt used	Vitamin	Salt used
 Neopyrithiamine	Hydrobromide	 Thiamine	Hydrobromide
Benzimidazole	—	 Adenine	—
 Desoxypyridoxine	Hydrochloride	 Pyridoxine	Hydrochloride
 4-Aminopteroylglutamic acid	Ammonium	 Pteroylglutamic acid	Ammonium
Pantothenol	—	 Pantothenic acid	Calcium
Pantoyltaurine	—		
 p-Aminobenzenesulphonamide	—	p-Aminobenzoic acid	—
p-Aminoacetophenone	—		
3-Acetylpyridine	—	Nicotinic acid	—
Pyridine-3-sulphonic acid	—		
dl-Pipecolinic acid	—		
iso-Nicotinic acid	—		
Avidin	—	Biotin	—

light cone to the dark area of the dish and could be recognized easily by their fast movements. Since the light made the black cephalo-pharyngeal skeleton particularly conspicuous, it was also possible to detect dead larvae.

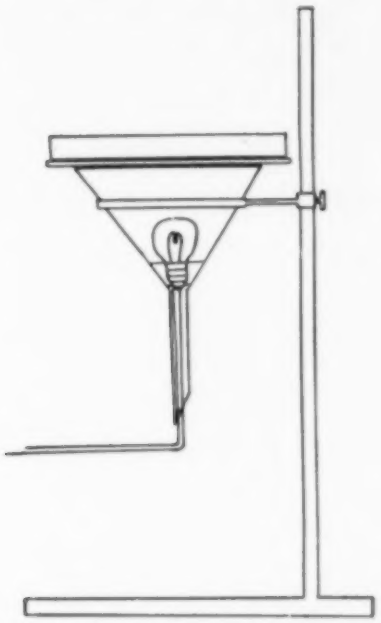


FIG. 1. Arrangement for the detection of retarded larvae in the rearing medium.

RESULTS AND DISCUSSION

The influence of antivitamins added in gradually increasing levels to the larval diet is shown by the results given in Table 3, in which the total number of larvae used in these experiments is indicated for each concentration of antivitamin. All antagonists retarded, proportionally to their concentration, larval growth and consequently reduced the percentage of pupae and adult flies. In presence of the highest applied levels of neopyrithiamine, desoxypyridoxine, 4-aminopteroylglutamic acid, pantothenol, 3-acetylpyridine, α-picolinic acid, and *p*-aminoacetophenone, the surviving larvae attained less than 10 per cent of the body weight gained normally within 72 hr of growth. With suitable amounts of pyridine-3-sulphonic acid and benzimidazole, the surviving larvae reached a slightly higher weight than for the above antivitamins, but then their growth stopped entirely, whereas with sublethal concentrations of *p*-aminobenzene-sulphonamide and *iso*nicotinic acid larval growth was suppressed only moderately. Newly hatched larvae died several hours after being placed on diets containing the latter two compounds at the highest concentration tabulated.

Larval growth was reduced considerably by egg albumen applied in its raw state, whereas with the heated preparation growth proceeded almost normally. This is undoubtedly due to the ability of avidine to form with biotin a heat-labile complex. Indeed, the effect of heating egg albumen could be duplicated by addition of a large dosage of biotin to the raw preparation.

TABLE 3—EFFECT OF GRADED AMOUNTS OF ANTIVITAMINS ON HOUSEFLY LARVAE

Antivitamin added to diet	Amount (mg/g dry diet)	No. of experimental animals	Larvae surviving at 72 hr (%)	Average larval weight at age 72 hr		Larvae pupating* (%)	Larvae developing into adults† (%)
				(mg)	as % of control		
Neopyrithiamine	0·06	60	100	17·9	92	93	90
	0·13	60	95	18·5	84	93	87
	0·27	56	89	18·1	82	80	73
	0·54	84	52	4·6	26	7	2
	1·09	80	39	0·8	5	0	—
	2·19	120	28	0·4	2·5	0	—
Desoxypyridoxine	0·27	60	100	18·7	100	93	90
	0·54	56	89	18·2	97	86	78
	1·09	58	96	15·9	85	90	83
	2·19	64	91	6·1	28	22	22
	4·38	53	75	3·9	20	4	2
	8·76	102	33	1·2	7	0	—
4-Aminopteroyl-glutamic acid	0·03	31	100	16·4	84	90	87
	0·06	30	100	13·9	71	87	77
	0·13	54	75	4·5	20	25	21
	0·27	60	67	2·4	11	0	—
	0·54	53	70	1·0	6	0	—
	1·09	114	32	0·4	2·5	0	—
Pantothenol	2·19	52	96	17·8	119	94	90
	4·38	54	92	13·3	89	85	74
	8·76	60	93	11·4	70	68	55
	13·12	66	82	10·6	53	42	40
	17·52	100	81	7·2	39	23	15
	35·04	95	36	1·8	9	0	—
3-Acetylpyridine	2·19	30	100	17·9	113	93	87
	4·38	28	100	17·8	113	90	80
	8·76	50	98	17·7	108	80	73
	13·12	84	45	5·4	30	5	1
	15·33	59	14	1·4	7	0	—
	17·52	90	0	—	—	—	—
α-Picolinic acid	2·19	57	100	20·8	100	93	93
	4·38	55	100	18·6	95	89	86
	8·76	60	97	10·9	61	80	66
	17·52	116	66	2·7	17	0	—
	35·04	30	27	0·5	3	0	—
*Iso*nicotinic acid	4·38	25	80	16·1	90	80	76
	8·76	55	24	11·3	63	16	12
	17·52	60	0	—	—	—	—

* Including abnormal pupae. † Including partially emerged flies.

TABLE 3—*continued*

Antivitamin added to diet	Amount (mg/g dry diet)	No. of experimental animals	Larvae surviving at 72 hr (%)	Average larval weight at age 72 hr (mg)	Average larval weight at age 72 hr as % of control	Larvae pupating* (%)	Larvae developing into adults† (%)
Pyridine-3-sulphonic acid	4·38	61	100	19·3	97	96	88
	8·76	63	98	18·6	99	90	82
	17·52	62	97	18·1	100	83	80
	35·04	64	88	14·0	78	73	67
	52·58	60	33	4·9	25	0	—
	70·08	57	18	2·8	14	0	—
p-Aminobenzene-sulphonamide	4·38	32	100	12·0	78	87	56
	6·57	54	85	8·2	46	41	9
	8·76	91	41	5·4	35	3	0
	17·52	60	0	—	—	—	—
p-Aminoaceto-phenone	4·38	30	40	8·2	53	30	3
	8·76	62	37	4·5	29	7	0
	17·52	30	10	1·0	7	0	—
	35·04	30	0	—	—	—	—
Benzimidazole	2·74	30	80	20·8	110	80	73
	5·48	30	73	16·9	90	70	50
	10·96	90	37	5·2	28	2	0
	16·44	61	37	3·0	16	0	—
	21·90	63	0	—	—	—	—
	Amount (ml/g dry diet)						
Egg albumen, fresh	2·2	60	37	5·6	33	0	—
Egg albumen, heated	2·2	60	90	15·0	89	67	61
Egg albumen, fresh + Biotin	2·2 0·5 mg	30	83	14·1	83	70	53
No addition	—	30	97	16·9	100	80	73

* Including abnormal pupae. † Including partially emerged flies.

It is rather interesting that both the α- and the γ-isomer of nicotinic acid as well as 3-acetylpyridine had about the same activity, whereas pyridine-3-sulphonic acid was approximately four times less active.

Pantoyltaurine did not affect larval growth at a level of 2·2 mg/g dry diet, whereas at amounts of 4·4 and 8·7 mg the respective larval weights were 19 and 70 per cent less than the control value.

The observed dietary levels of antivitamins which inhibited growth cannot, of course, be regarded as real threshold values, because the amount of antagonist ingested by each larva is unknown. Relatively high concentrations of antivitamins

TABLE 4—RESPONSE OF HOUSEFLY LARVAE TO GRADED AMOUNTS OF VITAMINS IN PRESENCE OF ANTIVITAMINS

Antivitamin added	Mg/ diet*	Vitamin added	Mg/ diet*	Ratio	Average larval weight at age 72 hr (mg)	Larvae surviving at 72 hr (%)	Larvae pupating (%)
Neopyrithiamine	0·0	Thiamine	0·0	—	14·0	83	70
	10·0		0·0	—	0·5	20	0
	10·0		0·001	10000/1	0·5	43	0
	10·0		0·01	1000/1	0·8	47	0
	10·0		0·1	100/1	9·4	80	37
	10·0		0·2	50/1	13·3	73	53
	10·0		0·4	25/1	15·7	86	66
	10·0		1·0	10/1	16·9	87	80
	10·0		10·0	1/1	17·2	94	91
	10·0		100·0	0·1/1	17·4	90	78
	0·0		100·0	—	18·5	100	100
Desoxypyridoxine	0·0	Pyrid-	0·0	—	19·4	90	90
	40·0	oxine	0·0	—	1·0	36	0
	40·0		0·004	10000/1	1·1	53	0
	40·0		0·04	1000/1	1·2	50	0
	40·0		0·4	100/1	4·7	69	0
	40·0		0·8	50/1	6·5	63	10
	40·0		4·0	10/1	11·6	70	23
	40·0		40·0	1/1	18·7	80	67
	40·0		400·0	0·1/1	0·2	7	0
	0·0		400·0	—	—	0	—
Pantothenol	0·0	Pantothe-	0·0	—	19·8	77	74
	160·0	nic acid	0·0	—	0·8	22	0
	160·0		0·016	10000/1	1·0	25	0
	160·0		0·16	1000/1	1·8	27	0
	160·0		1·6	100/1	4·1	33	0
	160·0		16·0	10/1	4·2	47	0
	160·0		32·0	5/1	4·6	61	7
	160·0		160·0	1/1	7·5	60	10
	160·0		320·0	0·5/1	6·9	67	13
	160·0		640·0	0·25/1	3·1	27	0
	160·0		960·0	0·17/1	0·7	10	0
	0·0		960·0	—	1·0	13	0
4-Aminopteroyl-glutamic acid	0·0	Folic acid	0·0	—	16·2	93	70
	5·0		0·0	—	0·4	33	0
	5·0		0·1	50/1	2·5	37	0
	5·0		1·0	5/1	2·7	44	0
	5·0		10·0	0·5/1	4·1	50	0
	5·0		100·0	0·05/1	7·7	90	10
	5·0		200·0	0·025/1	5·8	72	7
	0·0		200·0	—	17·1	57	50

* Wet weight of diet = 26·116 g.

TABLE 4—*continued*

Antivitamin added	Mg/ diet*	Vitamin added	Mg/ diet*	Ratio	Average larval weight at age 72 hr (mg)	Larvae surviving at 72 hr (%)	Larvae pupating (%)
Benzimidazole	0·0	Adenine	0·0	—	14·3	73	60
	50·0		0·0	—	3·9	57	3
	50·0		0·005	10000/1	3·6	71	0
	50·0		0·05	1000/1	2·8	77	0
	50·0		0·5	100/1	3·7	63	0
	50·0		5·0	10/1	3·8	80	7
	50·0		50·0	1/1	3·3	67	0
	50·0		500·0	0·1/1	—	0	0
	0·0		500·0	—	0·4	17	0
3-Acetylpyridine	0·0	Nicotinic acid	0·0	—	16·5	93	83
	60·0		0·0	—	4·1	39	0
	60·0		0·006	10000/1	5·6	37	0
	60·0		0·06	1000/1	5·7	47	0
	60·0		0·6	100/1	6·0	47	3
	60·0		6·0	10/1	7·0	73	7
	60·0		12·0	5/1	5·7	27	3
	60·0		24·0	25/1	4·7	23	0
	60·0		36·0	1·67/1	1·3	10	0
	60·0		48·0	1·25/1	—	0	—
	60·0		600·0	0·1/1	—	0	—
	0·0		600·0	—	0·9	53	0
	70·0		0·0	—	1·5	10	0
	70·0		0·007–700·0	9 ratios	—	0	—
	80·0		0·0	—	—	0	—
	80·0		0·008–800·0	9 ratios	—	0	—
p-Aminobenzenesulphonamide	0·0	*p*-Aminobenzoic acid	0·0	—	15·2	80	73
	40·0		0·0	—	6·2	34	0
	40·0		0·004	10000/1	5·6	29	0
	40·0		0·04	1000/1	5·3	26	0
	40·0		0·4	100/1	6·3	40	0
	40·0		4·0	10/1	5·8	54	0
	40·0		40·0	1/1	1·6	54	0
	40·0		400·0	0·1/1	—	0	—
	0·0		400·0	—	0·5	23	0
p-Aminobenzenesulphonamide	0·0	Folic acid	0·0	—	15·6	93	87
	40·0		0·0	—	4·2	27	0
	40·0		0·004	10000/1	5·4	13	0
	40·0		0·04	1000/1	6·2	20	3
	40·0		0·4	100/1	7·9	26	3
	40·0		4·0	10/1	5·0	25	0
	40·0		40·0	1/1	4·2	15	0
	40·0		400·0	0·1/1	1·5	10	0
	0·0		400·0	—	6·1	20	0

* Wet weight of diet = 26·116 g.

VOL. 3 1959

were necessary to assure uniform distribution of these agents in the strongly absorbent paper powder.

In order to ascertain whether the action of the compounds tested on housefly larvae was indeed an antivitamin effect, the experiments summarized in Table 4 were carried out. They show the growth response of larvae to increasing amounts of vitamins in the presence of a constant level of the corresponding antivitamins. Each concentration or ratio has been tested on at least two separate groups of thirty larvae. The action of neopyrithiamine and desoxypyridoxine was completely reversed by addition of thiamine and pyridoxine at a ratio of 1 : 25 and 1 : 1 respectively. On the other hand, the effect of 4-aminopteroylglutamic acid, pantothenol, and 3-acetylpyridine was counteracted only partially by the corresponding vitamins. When the concentration of 3-acetylpyridine was increased beyond the lowest inhibitory level, its effect could no longer be reversed at all by nicotinic acid. The growth inhibition produced by benzimidazole could not be relieved by adenine, in spite of the fact that this purine acts as a growth factor for larvae of *M. vicina* (LEVINSON, 1958) as well as of *M. domestica* (BROOKES, 1957). The effect of *p*-aminobenzene-sulphonamide was not counteracted by *p*-aminobenzoic acid, although it was partly overcome by folic acid. The latter observation is somewhat surprising, as fly larvae neither synthesize folic acid (as evidenced by their need for this compound) nor require *p*-aminobenzoic acid. The growth inhibitory action of both analogues of *p*-aminobenzoic acid appears to be a non-specific toxic effect.

In cases in which the antivitamin action was reversed partially by the corresponding vitamin, the larval weight increased first to a maximum and decreased again upon further addition of the vitamin. As an explanation of this effect one could assume that the vitamins in relatively large doses are toxic for the larvae. This is, indeed, the case. Each vitamin, when fed alone at the maximum level employed in the titration of the antivitamins, proved toxic, with the exception of thiamine and—partly—folic acid which was not toxic at a level of 200 mg in the diet, but had a strong growth-inhibiting effect at the 400 mg level.

In the course of the titration of the antivitamins by the corresponding vitamins a number of pathological changes were observed in the larvae as well as in the pupae and adults reared from them. Thus, partial lack of either pyridoxine or nicotinic acid gave lethargic larvae which moved unusually slowly, lacked appetite, and suffered from heavy diarrhoea. They invariably died in a typically paralysed position (Fig. 2). The symptoms appeared to be rather specific for a deficiency of these two vitamins and resembled a disease of larvae of *Bombyx mori* which was named by PAILLOT (1930) 'dysenterie flaccidiforme'. A slight deficiency in any of the vitamins tested still permitted growth of the larvae but caused them to pupate abnormally. The larval contracture was either lacking or incomplete. In the latter case, transitory forms were obtained which lacked the typical oval shape of the puparium, but had a fully sclerotized and pigmented cuticle (Fig. 3). The uncontracted pupae never developed into adult flies (Fig. 3a, b), whereas the partly contracted pupae gave rise to flies which could not free themselves

VOL.
3
1959

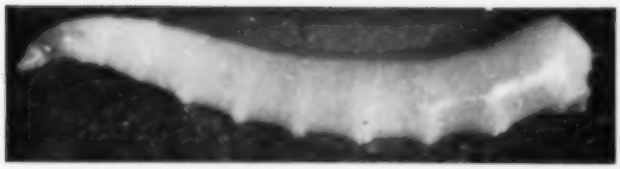


FIG. 2. Larva paralysed due to lack of pyridoxine or nicotinic acid in the diet.

(a) (b) (c) (d)

FIG. 3 (a–d). Abnormal forms of pupae, due to partial vitamin deficiencies.

FIG. 4. Housefly developed from larva suffering from slight pyridoxine deficiency.

(a) (b)

FIG. 5 (a, b). Flies developed from biotin-deficient larvae.

FIG. 6. Abnormally small flies produced by suboptimal vitamin levels in the larval diet.

FIG. 7. Dead female flies fed during lifetime with milk containing antivitamins (note the erected ovipositors).

from the puparia (Fig. 3c, d) and starved to death in this position. A number of flies unable to remove one of their wings from the pupal skin developed from larvae which suffered from a slight pyridoxine deficiency (Fig. 4), and four flies which could not spread their wings and were, therefore, unable to fly, were obtained from biotin-deficient larvae (Fig. 5). When the vitamin amounts in the diets were increased to suboptimal levels, the abnormal forms were replaced by very small flies (Fig. 6) which, however, behaved normally.

It can be concluded that housefly larvae require in their diet thiamine, pyridoxine, biotin, pantothenic, nicotinic, and folic acid, but not *p*-aminobenzoic acid. A larval requirement for riboflavine has been shown before; irradiation of yeast extract under alkaline conditions* produced dietary deficiency in this vitamin (LEVINSON, 1958). Thus, it was possible to replace the yeast extract of the artificial diet (Table 1) by a mixture of the six vitamins listed above and riboflavine, in addition to choline and RNA.† On the resulting synthetic diet, the larvae grew fairly well, but reached within a growth period of 72 hr only $\frac{2}{3}$ of the weight attained on the artificial diet. This would indicate that yeast extract provides the housefly larvae, in addition to the foregoing nutrients, with some other yet unknown growth factors.

VOL. 3 1959

The vitamin requirements of *Musca vicina* larvae thus differ very little from those of other insect larvae. All investigated species were found to require at least thiamine, riboflavine, pyridoxine, pantothenic, and nicotinic acid, some of them also choline, folic acid, and biotin; a few Tenebrionidae require carnitine (β-hydroxybutyrobetaine) in addition (FRAENKEL, 1951). Folic acid was reported not to be required for *M. domestica*, reared on a synthetic diet containing RNA (BROOKES, 1957), and for the larvae of *Lucilia sericata* (KADNER and LAFLEUR, 1951), *Pseudosarcophaga affinis* (HOUSE, 1954) and *Phormia regina* (BRUST and FRAENKEL, 1955). It was claimed that the first named species could also dispense with biotin and the second one with pyridoxine. However, it may well be that the methods used, contrary to the method of antivitamins, cannot detect requirements for *small* quantities of essential growth factors.

Antivitamins can also be used, as some preliminary experiments have shown, for the study of the vitamin requirements of the adult housefly. This method is less laborious than the usual one which requires both larvae and imagos to be kept under sterile conditions.

Equal numbers of both sexes of *M. vicina* were allowed to feed on sugar and an aqueous suspension of 20 per cent (w/v) powdered milk containing the antivitamin to be tested. This suspension was renewed daily throughout the lifetime of the insects. When given sugar and milk only, the average number of eggs laid by a female during life amounted to 470 (T 28°C, RH 65 per cent). This number was decreased by 82–99 per cent upon addition of 2·5 per cent pantothenol,

* Lumiflavine has no vitamin activity for housefly larvae.

† The vitamin requirements of *M. domestica* larvae, determined recently by the conventional (omission) method (BROOKES and FRAENKEL, 1958; HOUSE and BARLOW, 1958), appear to agree substantially with the results recorded above.

0·3 per cent neopyrithiamine, 0·2 per cent desoxypyridoxine, or 0·02 per cent 4-aminopteroylglutamic acid to the milk. Oviposition became totally inhibited when the flies were fed on milk containing 5·1 per cent of pantothenol, 1·3 per cent 3-acetylpyridine, or 0·3 per cent of desoxypyridoxine. It should be emphasized that in spite of normal copulation, the ovaries remained in the same atrophied state as the ones of females which had no access to protein (ASCHER and LEVINSON, 1956). The average diameter (breadth) of these ovaria was 0·6 mm as compared with 2·4 mm of the controls. However, since these flies obtained an ample supply of milk, the antivitamins apparently impeded the protein utilization necessary for ovarian development. The antivitamins also reduced significantly the longevity of both sexes. The females died mostly in a bizarre position with erected ovipositors just as for oviposition, their ovaria contained no eggs and their alimentary tracts were filled with undigested milk (see Fig. 7).

These results indicate that houseflies need for reproduction in addition to sugar, protein, and water, also vitamins. To what extent vitamins stored by the larvae are transferred to the adult flies has to be investigated further.

Also in a few other insects, certain vitamins were found to be indispensable to egg formation. Thus, *Cimex lectularius* did not lay eggs when fed on thiamine-deficient rats (DEMEILLON and GOLBERG, 1947); ovarian development of *Phormia regina* was accelerated significantly by addition of nicotinic acid to a diet consisting of protein and sugar solution (RASSO and FRANKEL, 1954) and egg formation was inhibited in *Drosophila melanogaster* (GOLDSMITH and FRANK, 1952) and *Musca domestica* (MITLIN, BUTT, and SHORTINO, 1957) by addition of 4-aminopteroylglutamic acid to their food.

REFERENCES

ASCHER K. R. S. and LEVINSON Z. H. (1956) The influence of protein addition to the larval diet on oviposition of the housefly. *Riv. Parassit.* **17**, 217–222.

BEAN W. B., SPIES T. D., and BLANKENHORN M. A. (1944) *Medicine* **23**, 1, cited by MARTIN G. J. (1951), *Biological antagonism*, Blakiston Co., New York, p. 318.

BHATTACHARYYA P. K., FRIEDMANN S., and FRAENKEL G. (1954) The effect of some derivatives and structural analogs of carnitine on the nutrition of *Tenebrio molitor*. *Arch. Biochem. Biophys.* **54**, 424–431.

BROOKES V. J. (1957) Ph.D. thesis, University of Illinois, Urbana, U.S.A.

BROOKES V. J. and FRAENKEL G. (1958) The Nutrition of the Larva of the Housefly *Musca domestica* L. *Physiol. Zool.* **31**, 208–223.

BRUST M. and FRAENKEL G. (1955) The nutritional requirements of the larvae of a blowfly, *Phormia regina* (Meig.). *Physiol. Zool.* **28**, 186–204.

CRAVENS W. W. (1952) Vitamin deficiencies and antagonists. *Ann. N.Y. Acad. Sci.* **55**, 188–195.

DEMEILLON B. and GOLBERG L. (1947) Nutrition of *Cimex* (Hemiptera) and *Ornithodorus* (Acarina). *J. exp. Biol.* **24**, 41–63.

FRAENKEL G. (1951) Isolation procedures and certain properties of vitamin B.T. *Arch. Biochem. Biophys.* **34**, 468–476.

GOLDSMITH E. C. and FRANK I. (1952) Sterility in the female fruitfly, *Drosophila melanogaster*, produced by the feeding of a folic acid antagonist. *Amer. J. Physiol.* **171**, 726–727.

GOLDSMITH E. C., HARNLY M. H., and TOBIAS E. B. (1950) Folic acid analogues in lower animals. I. The Insecta: *Drosophila melanogaster*. *Ann N.Y. Acad. Sci.* **52**, 1342–1345.

HINTON T. (1952) A quantitative study of folic acid requirements and reversal of aminopterin inhibition in *Drosophila*, *Science* **116**, 708–710.

HOUSE H. L. (1954) Nutritional studies with *Pseudosarcophaga affinis* (Fall.) a dipterous parasite of the spruce budworm, *Choristoneura fumiferana* (Chem.). II. Effects of eleven vitamins on growth. *Canad. J. Zool.* **32**, 342–350.

HOUSE H. L. and BARLOW J. S. (1958) Vitamin requirements of the Housefly, *Musca domestica* L. (Diptera, Muscidae) *Ann. ent. Soc. Amer.* **51**, 299–302.

KADNER C. G. and LAFLEUR F. M. (1951) The vitamin requirements of *Phaenicia sericata* (Meig.) larvae (Diptera: Calliphoridae). *Wasmann J. Biol.* **9**, 129–133.

LEVINSON Z. H. and BERGMANN E. D. (1957) Steroid utilization and fatty acid synthesis by the larva of the housefly *Musca vicina* (Macq.). *Biochem. J.* **65**, 254–260.

LEVINSON Z. H. (1958) Ph.D. thesis, University of Jerusalem, Israel.

MCILWAIN H. (1940) Pyridine-3-sulphonic acid and its amide as inhibitors of bacterial growth. *Brit. J. exp. Path.* **21**, 136–147.

MITLIN N., KONECKY M. S., and PIQUETT P. G. (1954) The effect of a folic acid antagonist on the housefly. *J. econ. Ent.* **47**, 932–933.

MITLIN N., BUTT B. A., and SHORTINO T. J. (1957) Effect of mitotic poisons on housefly oviposition. *Physiol. Zool.* **30**, 133–136.

MOELLER E. F. and BIRKOFER L. (1942) Konstitutionsspezifität der Nikotinsäure als Wuchsstoff bei *Proteus vulgaris* and *Streptobacterium plantarum*. *Ber. dtsch. chem. Ges.* **75**, 1108–1118.

OLESON J. J., HUTCHINGS B. O., and SUBBAROW Y. (1948) Studies on the inhibitory nature of 4-aminopteroylglutamic acid. *J. Biol. Chem.* **175**, 359–365.

OTT W. H. (1946) Antipyridoxine activity of 2,4-dimethyl-3-hydroxy-5-hydroxy-methylpyridine in the chick. *Proc. Soc. exp. Biol. N.Y.* **61**, 125–127.

PAILLOTT A. (1930). *Traité de maladies du ver à soie*. G. Doin et Cie., Paris.

RASSO S. C. and FRAENKEL G. (1954) The food requirements of the adult female blowfly, *Phormia regina* (Meig.), in relation to ovarian development. *Ann. ent. Soc. Amer.* **47**, 636–645.

SHYAMALA M. B. and BHAT J. V. (1958) Antimetabolites in the nutrition of the silkworm *Bombyx mori* L. I. Niacin and pyridoxin antagonists. *J. Ins. Physiol.* **2**, 137–141.

SIEBURTH J. F. and MCLAREN B. A. (1953) Growth studies with the cockroach, *Periplaneta americana* (Linn.), fed vitamin-deficient diets substituted with corresponding anti-vitamins. *Ann. ent. Soc. Amer.* **46**, 43–48.

SNELL E. E. and SHIVE W. (1945) Growth inhibition by analogues of pantothenic acid, pantothenyl alcohol and related compounds. *J. Biol. Chem.* **158**, 551–559.

WOOLLEY D. W. (1944) Some biological effects produced by benzimidazole and their reversal by purines. *J. Biol. Chem.* **152**, 225–232.

WOOLLEY D. W. (1952) *A Study of Antimetabolites*. J. Wiley, New York.

WOOLLEY D. W. and COLLYER M. L. (1945) Production of nicotinic acid deficiency with 3-acetyl-pyridine, the ketone analogue of nicotinic acid. *J. Biol. Chem.* **157**, 455–457.

WOOLLEY D. W. and WHITE A. G. C. (1943) Production of thiamine deficiency disease by the feeding of a pyridine analogue of thiamine. *J. Biol. Chem.* **149**, 285–289.

VOL. 3 1959

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 306 *to* 322. *Pergamon Press Ltd., London. Printed in Great Britain*

DISTRIBUTION AND SUBSTRATE SPECIFICITY OF ESTERASES IN THE HOUSEFLY, *MUSCA DOMESTICA* L.

K. VAN ASPEREN*

Lab. voor Insekticidenonderzoek, Vondellaan 6 Utrecht, Netherlands

(*Received* 29 *December* 1958)

Abstract—Housefly homogenates perform high cholinesterase and ali-esterase activity. Warburg-manometric studies show that acetylcholine, acetyl-β-methylcholine, butyrylcholine, and benzoylcholine are exclusively hydrolysed by a cholinesterase, the properties of which are more or less comparable to those of true vertebrate cholinesterase. Methylbutyrate and tributyrin are hydrolysed by one or possibly several ali-esterases but not by the cholinesterase. The cholinesterase activity is mainly present in the head and the ali-esterase activity in the thorax and the abdomen. Various other substrates, e.g. methylacetate, ethylacetate, amylacetate, triacetin, phenylacetate, and *o*-nitrophenylacetate are hydrolysed by both the cholinesterase and the ali-esterases. There is some evidence that *o*-nitrophenylacetate hydrolysis is partly due to an organophosphate-resistant enzyme distinct from the cholinesterase and ali-esterases, which are both inhibited by low concentrations of organophosphates. The ali-esterase activity is rapidly destroyed at 37°C in slightly alkaline media, whereas the cholinesterase is not affected by these conditions.

INTRODUCTION

THE presence of acetylcholine and of the enzymes for its synthesis and hydrolysis in several insect species is well established (CHADWICK and HILL, 1947; LEWIS, 1953; LEWIS and SMALLMAN, 1956; CHEFURKA and SMALLMAN, 1956; SMALLMAN, 1956, 1958). It has been suggested that, as in mammals, the insecticidal action of organophosphorus compounds is due to their anticholinesterase properties (METCALF, 1955; METCALF, FUKUTO, and MARCH, 1958) and consequent disturbances in the levels and distribution of substrate (LEWIS and FOWLER, 1956; SMALLMAN, 1958; SMALLMAN and FISCHER, 1958; COLHOUN, 1958). There is, however, also evidence that other esterases than cholinesterases may be important in organophosphate poisoning of insects (LORD and POTTER, 1950, 1951, 1954; POTTER *et al.*, 1957; HOPF, 1952, 1954; VAN ASPEREN, 1957, 1958) and this prompted the present study on esterase activities in the adult housefly.

METHODS AND MATERIAL

All the experiments were done with a normal strain of *Musca domestica* susceptible to insecticides. It was reared in the laboratory on a milk powder yeast diet at 25°C and 70 per cent relative humidity. Tests were made using homogenates of whole flies, heads, thoraces or abdomens. No attempt was made to purify the

* A part of the experimental work, especially that on the hydrolysis of *o*-nitrophenylacetate, was carried out by Mr. C. H. JANSSEN.

enzymes. The following substrates were added to give the initial concentrations indicated in the tables: acetylcholine, acetyl-β-methylcholine, butyrylcholine, benzoylcholine, methylacetate, ethylacetate, amylacetate, phenylacetate, *o*-nitrophenylacetate, triacetin, methylbutyrate, ethylbutyrate, and tributyrin. An attempt was made to characterize the enzymes—(i) by their distribution between the head and the rest of the insect body, (ii) by their sensitivities to inhibition by DDVP,* paraoxon,† and eserine, or (iii) by total activities in the presence of single and pairs of substrates.

Esterase activity was usually determined by the Warburg manometric method at 27° or 37°C in a total volume of 2·2 ml of solution, containing fly tissue and substrate at the concentrations required, 0·5 M NaCl and 0·025 M $NaHCO_3$. The high concentration of NaCl, according to CHADWICK, LOVELL, and EGNER (1953) and our own experiments proved to be favourable for the cholinesterase activity but not for the ali-esterase activity. In some experiments using methylbutyrate and tributyrin as substrates, therefore, 0·5 M NaCl was replaced by 0·15 M NaCl. In our view no striking differences may be expected from changing the NaCl-concentration. A gas mixture containing 95 per cent N_2 and 5 per cent CO_2, was passed over the reaction fluid for some minutes before closing the manometers and flasks, the pH during the measurement of hydrolysis and during the incubation of homogenates with inhibitors was approximately 7·5.

In some experiments the cholinesterase activity was measured by the colorimetric determination of acetylcholine as described by HESTRIN (1949). The hydrolysis of *o*-nitrophenylacetate was measured by the colorimetric estimation of *o*-nitrophenol, as described by HOPF (1954). If one of the two colorimetric methods was used the enzymatic reaction took place in a physiological salt solution containing 0·01 or 0·1 M phosphate buffer pH 6·9, 0·8 per cent NaCl, 0·04 per cent KCl and 0·032 per cent $MgSO_4$. As the yellow colour of *o*-nitrophenol is strongly dependent on pH, dilution of the sample for use in the colorimeter cell was made with 0·1 M phosphate buffer pH 6·9 and the colour developed compared with the colour obtained with known amounts of *o*-nitrophenol at the same pH. This procedure was especially necessary in those experiments where the hydrolysis caused a considerable change of pH during the reaction, e.g. in those cases where 0·01 M phosphate buffer was used and acid production was high.

As it was found in the course of our investigations that part of the esterase activity is extremely heat-labile, especially in alkaline media, the conditions under which the homogenates were prepared and kept will be mentioned wherever this may be important for the interpretation of the results. The conditions used will be indicated by the letters *a*, *b* and *c*, which are explained below.

(*a*) Flies or parts of flies were homogenized in a small type Waring blendor in 25 mM bicarbonate-Ringer solution without cooling. The homogenates obtained were kept at room temperature until use in the Warburg-experiments, which were performed at 37°C.

(*b*) Flies or parts of flies were homogenized in a small type Waring blendor in water or neutral salt solution with cooling in ice-water. The flasks containing the homogenates were kept in ice-water until use. In these experiments bicarbonate was mostly added at the very last moment before measurement of hydrolytic activity, which took place at 27°C.

* DDVP = O,O-dimethyl O-2,2 dichlorovinyl phosphate.

† paraoxon = O,O-diethyl O-*p*-nitrophenyl phosphate.

VOL. 3 1959

(*c*) Flies or parts of flies were homogenized in a mortar in phosphate buffer pH 6·9, and hydrolytic activity was measured colorimetrically as described above.

Although no exact measurements on the influence of temperature on hydrolytic rates were made, the available evidence points to an increase between 27°C and 37°C of approximately 50 per cent in the case of cholinester-splitting and of less than that in the case of methylbutyrate- and tributyrin-hydrolysis. This should be taken into account whenever hydrolysis-rates obtained under *a*- and *b*-conditions are compared.

EXPERIMENTAL RESULTS

(*A*) *Relation between activity and substrate concentration*

The rates at which several of the substrates under investigation were hydrolysed by homogenates of heads, thoraces and abdomens, or whole flies are shown in Table 1. Data shown on the same horizontal line of the table were obtained with a single homogenate. It is, therefore, possible to draw pS (−log substrate

TABLE 1—HYDROLYTIC ACTIVITY OF HOMOGENATES
(Activities all expressed in μl CO_2/30 min)

Substrate	Homogenate	Conditions*	Substrate concentration (micromoles/litre)								
			1·9	3·8	7·5	15	30	60	120	240	emulsion
Acetylcholine	2 heads	*a*	103	110	112	106	91	76	—	—	—
Acetylcholine	3 thoraces	*a*	85	105	108	105	87	63	45	—	—
Acetyl-β-methylcholine	2 heads	*a*	15	21	24	30	30	26	—	—	—
Butyrylcholine	2 heads	*a*	50	61	74	77	70	62	—	34	—
Benzoylcholine	2 heads	*a*	4	5	5	6	5	5	—	—	—
Amylacetate	2 heads	*a*	5	8	16	35	—	—	—	—	62
Triacetin	2 heads	*a*	38	60	90	123	148	—	169	—	—
o-Nitrophenyl-acetate	2 heads	*a*	—	89†	—	—	—	—	—	—	—
Phenylacetate	1 fly	*b*	—	—	217‡	—	—	—	—	—	—
Methylbutyrate	1 fly	*b*	—	25	45	94	193	353	409	—	—
Ethylbutyrate	1 fly	*b*	9	21	40	85	177	—	—	—	351
Tributyrin	1 fly	*b*	—	—	—	—	—	—	—	—	106

* See Section 'Methods and Material'.

† Corrected for ionization of *o*-nitrophenol ($pK_{27°} = 7·04$); actual CO_2-production/30 min/2 heads = 156 μl.

‡ Actual concentration 9×10^{-3} M.

concentration) activity curves for many of the substrates studied. Comparison of hydrolysis-rates for different substrates along vertical lines in the table, however, is liable to error since the hydrolytic activity of individual homogenates may vary to some extent.

(*B*) *Distribution in the housefly body*

When two substrates are hydrolysed by the same enzyme it is reasonable to expect the distribution of the activities to these substrates over different parts of the insect body to be similar. This will not hold, of course, where one of these substrates is split by a second enzyme.

The experimental procedure used was simple. Flies were decapitated and the activities shown by homogenates of the heads and by homogenates of the trunk (this term indicates thorax, abdomen and all the appendages) were compared. In many experiments thorax and abdomen were also separated and their activities separately determined. The results can be found in Table 2.

TABLE 2—DISTRIBUTION OF HYDROLYTIC ACTIVITY
(All experiments were performed under *b*-conditions)

Substrate	Concentration moles/litre	Percentage of total activity			
		head	thorax	trunk*	abdomen
Acetylcholine†	$1{\cdot}5 \times 10^{-2}$	68	27	32	5
Acetyl-β-methylcholine	$1{\cdot}5 \times 10^{-2}$	72	—	28	—
Butyrylcholine	$1{\cdot}5 \times 10^{-2}$	67	—	33	—
Triacetin	5×10^{-2}	53	—	47	—
Phenylacetate	9×10^{-3}	34	—	66	—
Methylbutyrate	$4{\cdot}8 \times 10^{-2}$	21	41	79	38
Tributyrin	emulsion	22	36	78	42

* Trunk = thorax + abdomen.

† The data in the table were obtained with the Warburg-manometric method. They could be confirmed by experiments in which acetylcholine determinations were made by the colorimetric method described by HESTRIN (1949).

(*C*) *Inhibition-experiments with organophosphates and eserine*

A further means of distinguishing the enzymes may be achieved by the determination of the inhibition caused by different inhibitors. The inhibitors used are DDVP, paraoxon, and eserine. A few remarks on esterase inhibition by these compounds need to be made in advance, since there exist several misunderstandings on this subject, which may lead to misinterpretation of the experimental results.

The organophosphorus compounds are irreversible inhibitors. The inhibition by these substances will increase as long as free inhibitor is present. The inhibitor molecules will be distributed over different hydrolytic enzymes and, perhaps, other proteins, and the degree of inhibition finally obtained for different enzymes will depend on their respective affinities for the inhibitor molecules, the rate at which inhibition becomes irreversible, and the total amount of inhibitor added.

In our experiments (Table 3) the time available for the reaction between the inhibitor and the components of the homogenate is sufficiently long for the inhibitor to be substantially used. Under these circumstances proportionality between I_{50} and homogenate concentration is to be expected. The problems

concerned with the kinetics of enzyme inhibition by DDVP have recently been dealt with in detailed publications (VAN ASPEREN, 1958a; VAN ASPEREN and DEKHUIJZEN, 1958).

In contrast to the organic phosphates eserine is a reversible inhibitor which can be displaced from the enzyme by its substrate even after long incubation periods so that the relative affinities and concentrations of inhibitor and substrates are of paramount importance whereas the total amounts are of little consequence.

The following results were obtained:

(1) The hydrolysis of all substrates studied was strongly inhibited by DDVP and paraoxon. Under the experimental conditions used, the I_{50} values were always lower than 10^{-8} M and in many cases even lower than 10^{-9} M. Susceptibility to inhibition varies considerably, which makes direct comparison of I_{50} values obtained in different experiments unreliable. As might be expected the homogenates of whole flies in general require higher inhibitor concentrations than those of fly-heads to obtain a given inhibition.

Evidence was obtained that even high concentrations of DDVP and paraoxon are unable to give full inhibition of hydrolysis if *o*-nitrophenylacetate is used as a substrate. Only about 70 per cent inhibition of trunk homogenates was obtained with 10^{-7} or 10^{-6} M concentrations of these organophosphates. Probably, therefore, part of the hydrolysis of this substrate is brought about by some

TABLE 3—INHIBITION OF HYDROLYSIS BY DDVP

Incubation time 1·5 hr. Experiments 1–4 were performed under *a*-conditions; Experiment 5 was performed under *c*-conditions

Substrate	Homogenate	Activity (μl CO_2/30 min)	Inhibitor-concentration † and percentage inhibition				No. of experiment
	heads			%		%	
Acetylcholine	2·5	156	$1{\cdot}5 \times 10^{-9}$	62	3×10^{-9}	89	1
Butyrylcholine	2·5	91	$1{\cdot}5 \times 10^{-9}$	69	3×10^{-9}	96	
Acetylcholine	2	126	10^{-9}	56	2×10^{-9}	89	2
Triacetin	2	134	10^{-9}	54	2×10^{-9}	90	
Acetylcholine	2	129	10^{-9}	58			3
Acetyl-β-methylcholine	6	93	3×10^{-9}	51			
Benzoylcholine	12	31	6×10^{-9}	52			
Acetylcholine	3	181	$0{\cdot}9 \times 10^{-9}$	50			4
Amylacetate	3	94	$1{\cdot}0 \times 10^{-9}$	50			
Acetylcholine	1	1·6*	10^{-9}	54	2×10^{-9}	81	5
o-Nitrophenyl-acetate	1	0·9*	10^{-9}	59	2×10^{-9}	80	

* Activity expressed as μ moles/30 min.

† In moles/litre.

organo-phosphate-resistant enzyme. This confirms the findings of METCALF et al., (1955) as to the presence of an organophosphate insensitive 'aromatic esterase'.

(2) When two substrates are hydrolysed by the same enzyme, the inhibition caused by a certain amount of inhibitor will be equal, provided the homogenate concentrations used in the experiments are equal. The inhibition values were, therefore, compared for pairs of substrates (Table 3).

(3) Inhibition studies with eserine gave the following results. The I_{50} value for the hydrolysis of acetylcholine proved to be approximately 5×10^{-9} M, whereas the I_{50} value for the hydrolysis of methylbutyrate and tributyrin was higher than 2×10^{-4} M. A large number of experiments were performed in which the inhibition of hydrolytic activities to different substrates by high concentrations of eserine (10^{-4}–10^{-6} M) was measured. The results are shown in Table 4.

TABLE 4—INHIBITION OF HYDROLYSIS BY ESERINE

Substrate	Concentration (moles/litre)	Homogenate	Conditions	Activity (μl CO_2/30 min)	Eserine-concentration (moles/litre)	Inhibition (per cent)	
Acetylcholine	$1{\cdot}5 \times 10^{-2}$	2 whole flies	*b*	100	10^{-5}	97	‡
Acetyl-β-methylcholine	$1{\cdot}5 \times 10^{-2}$	2 whole flies	*b*	28	10^{-5}	100	‡
Butyrylcholine	$1{\cdot}5 \times 10^{-2}$	2 whole flies	*b*	70	10^{-5}	100	
Triacetin	5×10^{-2}	2 whole flies	*b*	186	10^{-5}	68	
Triacetin	5×10^{-2}	2 trunk*	*b*	91	10^{-5}	44	
Amylacetate	emulsion	2 whole flies	*b*	114	10^{-5}	49	‡
Methylacetate	2×10^{-1}	2 whole flies	*b*	79	10^{-5}	8	‡
Ethylacetate	2×10^{-1}	2 whole flies	*b*	66	10^{-5}	15	
Phenylacetate	9×10^{-3}	1 whole fly	*b*	215	10^{-5}	20	
Phenylacetate	9×10^{-3}	1 trunk*	*b*	143	10^{-5}	17	‡
Phenylacetate	9×10^{-3}	2 heads	*b*	149	10^{-5}	64	‡
o-Nitrophenylacetate	$3{\cdot}6 \times 10^{-3}$	1 trunk*	*a*	69	10^{-6}	25	
o-Nitrophenylacetate	$3{\cdot}6 \times 10^{-3}$	2 heads	*a*	90	10^{-6}	87	‡
o-Nitrophenylacetate	$3{\cdot}6 \times 10^{-3}$	1 abdomen	*a*	21	10^{-6}	31	
o-Nitrophenylacetate	2×10^{-3}	0·4 heads	*c*	0·6†	10^{-6}	69	‡
o-Nitrophenylacetate	2×10^{-3}	0·4 trunk*	*c*	1·1†	10^{-6}	15	‡
o-Nitrophenylacetate	4×10^{-3}	0·5 heads	*c*	0·4†	10^{-6}	88	‡
o-Nitrophenylacetate	4×10^{-3}	0·5 trunk*	*c*	1·1†	10^{-6}	23	‡
Methylbutyrate	$4{\cdot}8 \times 10^{-2}$	1 whole fly	*b*	352	10^{-5}	3	
Tributyrin	emulsion	3 trunk*	*a*	90	10^{-6}	12	‡
Tributyrin	emulsion	3 trunk*	*a*	90	10^{-4}	33	‡

* Trunk = thorax + abdomen.
† Activity expressed as μ moles/30 min.
‡ Data obtained in the same experiment.

(*D*) *Summation experiments*

Experiments in which one homogenate is allowed to act on a mixture of two substrates may be used to decide whether one enzyme acts on both substrates or, if there are two enzymes, one for each substrate. Due attention, however, should

be paid to possibilities such as cross-inhibition (GOLDSTEIN, 1954) and to the effect of substrate concentration. Lack of summation is evidence in favour of the occurrence of only one enzyme splitting both substrates, but it is no definite proof for it. Summation of activities may be considered as an indication for the presence of two enzymes only when near-saturation substrate concentrations are used.

The experimental results are to be found in the Tables 5 and 6.

TABLE 5—SUMMATION EXPERIMENTS WITH PAIRS OF SUBSTRATES

All experiments were under *a*-conditions, except Experiment 9 where *b*-conditions were used. All activities expressed in μl CO_2/30 min; concentrations in moles/litre.

								No. Experiment
2·4 heads	Acetylcholine-conc.	3×10^{-2}	$1{\cdot}5 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	—	—		1
	Butyrylcholine-conc.	—	—	$1{\cdot}5 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	3×10^{-2}		
	Activity	126	142	84	84	76		
2·4 heads	Acetylcholine-conc.	$3{\cdot}6 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	$3{\cdot}6 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	—		2
	Butyrylcholine-conc.	—	—	$1{\cdot}5 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$		
	Activity	29	161	24	94	95		
2·4 heads	Acetylcholine-conc.	$1{\cdot}2 \times 10^{-2}$	$4{\cdot}5 \times 10^{-3}$	$1{\cdot}2 \times 10^{-2}$	$4{\cdot}5 \times 10^{-3}$	—		3
	Butyrylcholine-conc.	—	—	$1{\cdot}5 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$		
	Activity	81	122	67	82	91		
2·4 heads	Acetylcholine-conc.	$1{\cdot}5 \times 10^{-2}$	—	—	$1{\cdot}5 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	4
	Benzoylcholine-conc.	—	$1{\cdot}5 \times 10^{-2}$	3×10^{-2}	$0{\cdot}4 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	3×10^{-2}	
	Activity	135	5	0	76	29	11	
2·4 heads	Acetylcholine-conc.	$1{\cdot}5 \times 10^{-2}$	—	—	$1{\cdot}5 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	5
	Acetyl-β-methylcholine-conc.	—	$1{\cdot}5 \times 10^{-2}$	3×10^{-2}	$0{\cdot}4 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	3×10^{-2}	
	Activity	151	48	50	152	112	88	
2·0 heads	Acetylcholine-conc.	$1{\cdot}5 \times 10^{-2}$	—	—	$1{\cdot}5 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	6
	Triacetin-conc.	—	$1{\cdot}5 \times 10^{-2}$	$7{\cdot}5 \times 10^{-2}$	$0{\cdot}4 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	$7{\cdot}5 \times 10^{-2}$	
	Activity	142	106	162	126	116	112	
2·0 heads	Acetylcholine-conc.	$1{\cdot}5 \times 10^{-2}$	—	—	$1{\cdot}5 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	7
	Methylbutyrate-conc.	—	$1{\cdot}5 \times 10^{-2}$	4×10^{-2}	$0{\cdot}5 \times 10^{-2}$	$1{\cdot}5 \times 10^{-2}$	4×10^{-2}	
	Activity	126	6	10	128	128	121	
2·0 heads	Acetylcholine-conc.	$1{\cdot}5 \times 10^{-2}$	—	$1{\cdot}5 \times 10^{-2}$				8
	Methylbutyrate-conc.	—	$1{\cdot}2 \times 10^{-1}$	$1{\cdot}2 \times 10^{-1}$				
	Activity	135	10	130				
2·5 heads	Acetylcholine-conc.	$1{\cdot}5 \times 10^{-2}$	—	$1{\cdot}5 \times 10^{-2}$				9
	Methylbutyrate-conc.	—	$4{\cdot}8 \times 10^{-2}$	$4{\cdot}8 \times 10^{-2}$				
	Activity	121	85	187				
2·0 heads	Acetylcholine-conc.	$1{\cdot}5 \times 10^{-2}$	—	$1{\cdot}5 \times 10^{-2}$				10
	Amylacetate-conc.	—	emulsion	emulsion				
	Activity	119	63	107				

The experiments shown in Table 6 allowed of a calculation of the hydrolysis of both substrates (acetylcholine (ACh) and *o*-nitrophenylacetate (NPA)) separately, since NPA-hydrolysis was measured by the colorimetric determination of *o*-nitrophenol and the total hydrolysis by the Hestrin-method. The part of the two substrates in the total hydrolysis is shown in the last vertical column of Table 6.

TABLE 6—SUMMATION EXPERIMENTS WITH ACETYLCHOLINE AND *o*-NITROPHENYLACETATE

Experiments performed under *c*-conditions.
All activities expressed in μ moles/30 min; concentrations in moles/litre

0·5 heads	*ACh-conc. †NPA-conc.	10^{-3} —	2×10^{-3} —	— 10^{-3}	— 2×10^{-3}	10^{-3} 10^{-3}
Experiment 1	Activity	0·72	0·85	0·57	0·60	0·76 (0·45 ACh; 0·31 NPA)
Experiment 2	Activity	0·90	1·16	0·70	0·70	1·08 (0·72 ACh; 0·36 NPA)

* ACh = Acetylcholine.
† NPA = *o*-Nitrophenylacetate.

VOL. 3 1959

(*E*) *Additional experimental observations*

Some observations which are of great importance for the present subject need be mentioned here, though it is hoped to publish a more detailed study in a separate paper.

TABLE 7—LOSS OF HYDROLYSING CAPACITY OF HOMOGENATES

Homogenates prepared in Bicarbonate-Ringer*
Substrates: MB = Methylbutyrate; TB = Tributyrin

Homogenate	Sub-strate	Activity† (μl CO_2/30 min)	Temperature (°C)‡	Percentage of activity left after			
				5 min	15 min	60 min	120 min
1 whole fly	MB	364	26	—	88	55	27
3 whole flies	TB	410	26	—	83	46	39
1 whole fly	MB	306	36	58	24	18	15
3 whole flies	TB	369	36	—	55	44	38

* Bicarbonate-Ringer contains 0·5 M NaCl and 0·025 M $NaHCO_3$; pH of the homogenate is approximately 8·3.
† Activities measured at 27°C and pH 7·5.
‡ Temperature at which the homogenates are kept for the periods indicated.

(1) The capacity of housefly homogenates to hydrolyse acetylcholine remains constant for at least several hours at 37°C. A homogenate of whole flies lost only 1 per cent of its hydrolysing activity to acetylcholine if kept 2 hr at 37°C. Keeping a homogenate for 24 hr at room temperature (*ca.* 20°C) caused only 4 per cent loss of activity.

The capacity of homogenates to split methylbutyrate or tributyrin, however, was found to decrease very rapidly (Table 7). Heating to 50°C reduced the hydrolytic activity of a whole fly homogenate to methylbutyrate (340 μl CO_2/30 min) to 69 per cent, 28 per cent, 16 per cent, and 4 per cent after 1, 2 4, and 8 min respectively. One minute heating to 100°C destroyed all activity.

All data as given refer to experiments in which the homogenates were prepared in physiological salt solutions containing 0·025 M sodium bicarbonate. These homogenates, therefore, were slightly alkaline. When 5 per cent CO_2–95 per cent N_2 gas mixture was passed over the 'bicarbonate-Ringer-homogenates' with consequent lowering of pH, the inactivation at 37°C was very much slower. The inactivation rate was also much lower when the homogenates were prepared in neutral salt solutions. Most probably, therefore, the rate of inactivation is strongly dependent upon the concentration of hydroxyl-ions in the medium. Almost no inactivation took place if methyl butyrate was added to the homogenate. This could be due to a protective effect of the substrate, but the hydrolysis of the methylbutyrate causes a rapid fall in pH which would also account for the increased stability.

These observations, made in the course of our experimental work on the present subject, induced us to change our techniques of preparing and keeping the homogenates as described before, see method (b) instead of method (a).

(2) A second point, which held our attention, was the absence of proportionality between homogenate concentration and hydrolytic activity in many experiments with methylbutyrate and tributyrin. At low homogenate concentrations the activity was much less than would be expected assuming proportionality. This may be illustrated by the results of some typical experiments on methylbutyrate hydrolysis. A whole fly homogenate when diluted 4 and 8 times showed only 7 per cent and 1 per cent of the activity of an undiluted sample (activity 287 μl CO_2/30 min) respectively. When flyhead homogenates containing 6, 4, 3, 2, and 1 heads per ml were compared the activities (μl CO_2/30 min) proved to be 416, 272, 196, 128, and 38 respectively. These findings were qualitatively confirmed in several other experiments. The degree of disproportion, however, varied a great deal.

No such disproportion has ever been found in experiments on the hydrolysis of choline-esters. This phenomenon has always been taken into account in those experiments in which distribution of activity over different parts of the insect body was studied.

DISCUSSION AND CONCLUSIONS

From the data given in Tables 2 and 4 on distribution in the insect body and on inhibition by eserine it is clear that at least two different enzymes must be concerned in the hydrolytic processes studied.

Four arguments may be adduced in support of the view that *all choline-esters are hydrolysed by one and the same enzyme, which in general has the characteristic*

properties of the specific cholinesterase as found in mammalian brain and erythrocytes (AUGUSTINSSON, 1950).

(1) *The hydrolysis of all choline-esters studied is inhibited by excess substrate.* The rather high activity to butyrylcholine as compared to acetylcholine constitutes a clear deviation from the main pattern of true mammalian cholinesterase and suggests the occurrence of a second, non-specific, cholinesterase, such as the pseudo-cholinesterase of mammalian blood plasma. This suggestion is supported by the finding of METCALF, MARCH, and MAXON (1955), that the pS-activity curve for the hydrolysis of butyrylcholine is S-shaped. The absence of auto-inhibition found by these authors was not confirmed, however, by WOLFE and SMALLMAN (1956) and is in contradiction with our own results.

(2) *The distribution of the hydrolysing activities to different choline-esters in the housefly body is strikingly similar* (Table 2). It is distinctly different from that found for the other esters mentioned in Table 2, particularly that for methylbutyrate and tributyrin.

(3) *The hydrolysis of different choline-esters is equally inhibited by DDVP and eserine* (see Table 3, experiments 1 and 3, and Table 4). A correct interpretation of the results of Experiment 3 (Table 3) should take account of the proportionality of the inhibitor concentrations required and the homogenate concentrations used. The hydrolysis of the choline-esters studied was invariably nearly 100 per cent inhibited by high concentrations of eserine (Table 4). In contrast with this the inhibition of the hydrolysis of all other substrates was much less. The data reported here irrefutably prove that no eserine-resistant enzymes are concerned with the hydrolysis of any of the choline-esters under investigation.

(4) *None of the summation-experiments on pairs of choline-esters showed any summation of hydrolytic activities* (see Table 5, experiments 1–5). As pointed out before this is no absolute proof that only one enzyme is involved, but it may be considered as strong evidence in favour of this view.

A serious drawback of the *a*-conditions in preparing the homogenates used in the experiments shown in Table 5 lies in the possible inactivation of esterases, such as those hydrolysing methylbutyrate and tributyrin. If such labile esterases should take part in the hydrolysis of any of the choline-esters, this would not necessarily appear from the results of the summation-experiments. The stability of the acetylcholine hydrolysing capacity of homogenates proves that no labile esterases are concerned with the splitting of this substrate.

The available evidence thus points to one single enzyme being responsible for the hydrolysis of all choline-esters. This enzyme will henceforth be indicated by the name *cholinesterase*.

Some further conclusions with regard to affinities between the cholinesterase and different choline-esters can be drawn from the data in Table 5.

(a) The first three experiments clearly show that butyrylcholine is hydrolysed more slowly than acetylcholine at substrate concentrations which are near optimal (see also Table 1). This must be due to a lower turnover number for butyrylcholine- than for acetylcholine-hydrolysis. This lower turnover number, however, is attended by a much higher affinity for the enzyme, as is clearly brought

out by the results of those experiments in which both substrates are used simultaneously. The hydrolytic rate then found is almost equal to that observed with butyrylcholine alone, indicating a nearly complete displacement of acetylcholine from the active surface of the enzyme by butyrylcholine, acting in this case as an inhibitor. This only occurs when both compounds are used at approximately optimal substrate concentrations. If the concentration of acetylcholine is raised, it is better able to compete with butyrylcholine and the hydrolysis rate is equal to or even lower than that for acetylcholine alone, which, as a consequence of autoinhibition, is then very low. These findings are in full agreement, at least qualitatively, with the findings of BERGMANN and SHIMONI (1953) concerning the electric eel cholinesterase.

(b) Unlike butyrylcholine, benzoylcholine is not hydrolysed at an appreciable rate. However, its affinity to the enzyme, like that of butyrylcholine, is distinctly higher than that of acetylcholine. From the results of Experiment 4 it may be calculated that 50 per cent inhibition of acetylcholine hydrolysis (at an acetylcholine concentration of $1{\cdot}5 \times 10^{-2}$ M) is obtained with approximately $0{\cdot}5 \times 10^{-2}$ M benzoylcholine. At equal concentrations of both choline-esters ($1{\cdot}5 \times 10^{-2}$ M) the inhibition amounts to 79 per cent, roughly indicating the ratio of the affinities for benzoylcholine and for acetylcholine to be about 4. A similar inhibition by benzoylcholine, though much weaker, has also been described by LEHMANN and SILK (1955) for human red cell cholinesterase.

(c) The results of Experiment 5 show that the affinity of acetyl-β-methylcholine to the cholinesterase is somewhat less than that of acetylcholine. The much lower rate of hydrolysis obtained with acetyl-β-methylcholine as compared to that with acetylcholine (see also Table 1) in our view, cannot be explained, however, by this rather small difference in affinity.

Methylbutyrate and tributyrin are hydrolysed by some enzyme other than the cholinesterase. The arguments in favour of this view may be summarized as follows:

(1) The distribution of the methylbutyrate- and tributyrin-splitting capacity in the body is distinctly different from that of the cholinesterase (see Table 2).

(2) The capacity of homogenates to split methylbutyrate and tributyrin is greatly reduced under conditions (temperature 37°C, slightly alkaline medium) which do not affect the activity of the cholinesterase.

(3) Eserine concentrations completely inhibiting the cholinesterase activity do not appreciably affect the hydrolytic breakdown of methylbutyrate and tributyrin (see Table 4).

This eserine-resistant and heat–alkali–labile enzyme will be referred to as the *ali-esterase* (METCALF, MAXON, FUKUTO, and MARCH, 1956).

The Experiments 7–9 of Table 5 give some information on the possibility of cross-inhibition, i.e. inhibition of the cholinesterase and the ali-esterase by each other's substrates. In the Experiments 7 and 8 the ali-esterase activity, most probably as a consequence of heat-alkali-inactivation (*a*-conditions), is very low, which greatly facilitates the interpretation of the results. These possibly point to a weak inhibition of the cholinesterase-activity by the presence of high

concentrations of methylbutyrate. In Experiment 9 (*b*-conditions) an obvious but incomplete summation of hydrolytic activities was observed. This points to a weak inhibition of either enzyme by the substrate of the other.

It may be questioned whether there is only one ali-esterase concerned with the hydrolysis of methylbutyrate and tributyrin. Heat-alkali-inactivation experiments not reported in this paper indicate that possibly two or even more enzymes are present with slightly different properties, for example with respect to substrate preference. In our view, this possibility is of only little importance for the present subject and we feel ourselves justified, therefore, to deal with the hydrolytic activity to methylbutyrate and tributyrin as if brought about by one ali-esterase.

Now that the occurrence of two esterases with distinctly different substrate specificities has been established, the problem arises as to whether it is possible to explain the hydrolysis of the other substrates studied by the action of these two esterases.

(1) *Methylacetate.* The hydrolysis-rate, even at very high concentrations, is low (Table 4). Only 8 per cent of the activity of whole fly homogenates is inhibited by 10^{-5} M eserine. This result could most easily be explained by ascribing a small part of the activity to the eserine-sensitive cholinesterase and the remainder to the action of the eserine-resistant ali-esterase. The activity of the cholinesterase to methylacetate must be very low.

(2) *Ethylacetate.* What has been said with respect to methylacetate also applies to ethylacetate (Table 4). Cholinesterase activity to this substrate is very low and accounts for a small part only of the total hydrolytic activity.

(3) *Amylacetate.* This is hydrolysed much faster than methyl- and ethylacetate (Table 4). Probably most of the activity reported for the heads (Table 1) is due to the cholinesterase, especially since we were unable to demonstrate any appreciable ali-esterase activity in the heads under the *a*-conditions used in this experiment. This view is confirmed by the data given in Table 3, Experiment 4 (equal I_{50} values for DDVP-inhibition for acetylcholine and amylacetate) and by those given in Table 5, Experiment 10 (no summation of activities to acetylcholine and amylacetate). The results of an eserine inhibition experiment on whole fly homogenates (Table 4, *b*-conditions), however, clearly show that only about one half of the total hydrolytic activity is due to the cholinesterase and the other half to the action of an eserine-resistant esterase, at least if an amylacetate-emulsion (saturated solution contains about $1{\cdot}4 \times 10^{-2}$ Mol/l., at 18°C) is used. It is still uncertain whether this latter esterase is identical with the ali-esterase mentioned before.

(4) *Triacetin.* This substrate is very well split by flyhead homogenates, at high concentrations even faster than acetylcholine (Table 1). The data obtained in the DDVP-inhibition experiment (Table 3, 2) and the substrate-summation experiment (Table 5, 6) suggest this activity to be mainly due to the cholinesterase. Calculation of the distribution in the body of the eserine-sensitive part of the hydrolytic activity from the data given in Table 4 shows that 68 per cent is present in the head and 32 per cent in the remainder of the body. This confirms the

VOL. 3 1959

idea that triacetin-hydrolysis is for a major part brought about by the cholinesterase. The data in Table 4 also show that part of the hydrolytic activity to triacetin must be due to some eserine-resistant esterase, for which the distribution between the heads and the remainder of the body can be calculated to be in the proportion of fifteen to eighty-five, which is reasonably similar to that found for the ali-esterase.

Summarizing, it can be concluded that, if triacetin is used in 5×10^{-2} M concentration, the hydrolysis is performed by *a*, the cholinesterase in the head, *b*, the cholinesterase in thorax and abdomen, *c*, the ali-esterase in the head, and *d*, the ali-esterase in thorax and abdomen, to the extent of 46, 22, 5 and 27 per cent respectively.

(5) *Phenylacetate.* This substrate has been intensively studied by LORD and POTTER (1954), who concluded that its hydrolysis in a number of insect species is brought about by an esterase which is distinctly different from the cholinesterase. Our experiments show that the hydrolysis of phenylacetate by housefly homogenates, even at rather low substrate concentrations, proceeds rapidly (Table 1) and that part of it is inhibited by 10^{-5} M eserine (Table 4). Calculation of the distribution of the eserine-susceptible activity gives values of 67 per cent for the heads and 33 per cent for thorax and abdomen. Apparently this activity is due to the cholinesterase. The major part of the hydrolysis by whole fly homogenates, however, has to be ascribed to an eserine-resistant enzyme, of which, according to calculation from the data in Table 4, 18 per cent is present in the head and 82 per cent in the remainder of the body. This suggests this enzyme to be identical with the ali-esterase mentioned before. Most probably, therefore, phenylacetate is split both by the cholinesterase and by the ali-esterase, the latter enzyme being, at least quantitatively, the most important.

(6) *o-Nitrophenylacetate.* HOPF (1954) studied the hydrolysis of this ester by homogenates of the thoracic nervous system of *Locusta migratoria.* He concluded that possibly one 'acetylesterase' is responsible for the breakdown of *o*-nitrophenylacetate and acetylcholine and found no evidence for the existence of a specific acetylcholinesterase.

The present investigations permit the following conclusions.

(*a*) The hydrolytic activity of flyhead homogenates to *o*-nitrophenylacetate is high (Table 1). Results of experiments with flyhead homogenates, not reported in this paper, point to a marked independence of the activity of the substrate concentration, e.g. lowering the concentration from 2×10^{-3} M to $2{\cdot}5 \times 10^{-4}$ M caused a decrease of only some 20 per cent or less of the hydrolytic rate. This shows that even at very low concentrations the hydrolytic enzyme is nearly saturated with the substrate. This is confirmed by the data given in Table 6 for the hydrolysis of *o*-nitrophenylacetate at 10^{-3} and 2×10^{-3} M concentrations.

(*b*) According to the data of Table 4 a part of the hydrolysis is performed by some eserine-sensitive enzyme. Calculation shows that 70 per cent of this enzyme is present in the head and 30 per cent in thorax and abdomen, which points to its identity with the cholinesterase. Further evidence for this view can be got

from Experiment 5 of Table 3 with flyheads, which shows about equal inhibition of acetylcholine- and *o*-nitrophenylacetate-hydrolysis by DDVP.

(*c*) There can be no doubt as to the occurrence of an eserine-resistant esterase splitting *o*-nitrophenylacetate, which according to the data in Table 4 (*c*-conditions) is mainly present in thorax and abdomen (calculation in one experiment gives a value of 83 per cent, in another 94 per cent). The hydrolysis in thorax and abdomen may thus be due to the ali-esterase mentioned before, but the evidence is not conclusive.

According to the data given in the previous section there also exists an organophosphate-resistant enzyme splitting *o*-nitrophenylacetate.

(*d*) The results of two summation experiments with head homogenates are shown in Table 6. The colorimetric techniques used there made it possible to determine the two substrates separately, since the sum of acetylcholine and *o*-nitrophenylacetate can be estimated by the Hestrin-method (equimolecular solutions of these two compounds give equal colour intensities in this method), whereas the amount of *o*-nitrophenylacetate can be obtained by a colorimetric determination of *o*-nitrophenol.

VOL. 3 1959

The competition of acetylcholine and *o*-nitrophenylacetate for the cholinesterase is clearly demonstrated by the distinctly lower acetylcholine-hydrolysis in the presence of *o*-nitrophenylacetate. According to the inhibition of the hydrolysis of *o*-nitrophenylacetate by acetylcholine the same applies to the enzyme splitting *o*-nitrophenylacetate. It is probable, therefore, that both substrates are hydrolysed by the same enzyme—cholinesterase.

Adding up all the evidence on *o*-nitrophenylacetate, the most plausible conclusion seems to be that the hydrolysis of *o*-nitrophenylacetate in flyhead homogenates is largely performed by the cholinesterase and that in homogenates of thoraces and abdomens mainly by some other enzyme which possibly is identical with the ali-esterase.

(7) *Ethylbutyrate*. The rate of hydrolysis by whole fly homogenates proved to be about similar to that of methylbutyrate at equimolecular concentrations (see Table 1). It is believed that ethylbutyrate splitting is accomplished by the ali-esterase, but experimental proof for this view is lacking.

Surveying the results obtained with the seven substrates, the following statement can be made. All acetic acid esters studied are hydrolysed by the cholinesterase. The hydrolytic rate is low for the short chain acetates, such as methyl- and ethylacetate, but increases with increasing size of the alcoholic (or phenolic) portion of the substrate molecule. This may possibly be explained by assuming that the cholinesterase prefers those substrates, of which the dimensions are approximately equal to those of acetylcholine. The affinity of the cholinesterase for *o*-nitrophenylacetate is remarkably high and may be about equal to that for acetylcholine. Though arguments may be adduced for using the term 'acetylesterase' (Hopf, 1954) rather than cholinesterase, we believe that the latter is preferable on historical grounds as well as for the reason that choline-esters, including butyrylcholine, are exclusive substrates with very high affinities to this enzyme.

Although the ali-esterase found in houseflies may provisionally be defined as the enzyme capable of splitting methylbutyrate, it must be stressed that much higher rates of hydrolysis are obtained with phenylacetate, at least if comparison is made at the same molar substrate concentrations. As outlined above, several other acetates are also appropriately split by the ali-esterase.

Summing up once more it seems justifiable to ascribe most of the hydrolysis of all substrates studied to the action of two enzymes, which may be indicated by the names *cholinesterase* and *ali-esterase*.

SUMMARY

The hydrolytic activity of homogenates of whole houseflies or parts of houseflies has been investigated. The substrates used were: acetylcholine, acetyl-β-methylcholine, butyrylcholine, benzoylcholine, methylacetate, ethylacetate, amylacetate, triacetin, phenylacetate, *o*-nitrophenylacetate, methylbutyrate, ethylbutyrate, and tributyrin.

Most probably the hydrolytic activities towards the different substrates studied can be explained by the action of two main enzymes, a cholinesterase and an ali-esterase. The properties of the cholinesterase are more or less comparable to those of the so-called true cholinesterase as found in the nervous system and red blood cells of vertebrates. It is completely inhibited by 10^{-6} M concentrations of eserine, whereas the ali-esterase is eserine-resistant. The inhibition by eserine thus constitutes a reliable means for distinguishing the two enzymes. It was further shown that the ali-esterase is rapidly inactivated at 37°C in slightly alkaline media, e.g. 0·025 M bicarbonate-solution in air, whereas the cholinesterase is not affected by these conditions.

The following conclusions can be drawn:

(1) The distribution in the housefly of the cholinesterase and the ali-esterase is distinctly different. The cholinesterase is mainly found in the head (70 per cent) and much less in thorax (25 per cent) and abdomen (5 per cent). The ali-esterase is mainly found in thorax (*ca.* 40 per cent) and abdomen (*ca.* 40 per cent), and for a minor part only in the head (*ca.* 20 per cent).

(2) All choline-esters studied are hydrolysed by the cholinesterase only. Hydrolytic rates decrease in the order acetylcholine, butyrylcholine, acetyl-β-methylcholine, and benzoylcholine. Butyrylcholine and benzoylcholine show greater affinity to the enzyme than acetylcholine, whereas the affinity of acetyl-β-methylcholine proved to be somewhat lower than that of acetylcholine. Inhibition by excess substrate was shown to occur for the hydrolysis of acetylcholine, butyrylcholine and acetyl-β-methylcholine. The rate of hydrolysis of benzoylcholine was so small that no reliable results as to the occurrence of auto-inhibition could be obtained.

(3) Methylbutyrate and tributyrin are hydrolysed by the ali-esterase only. There may possibly be two or even more ali-esterases with slightly different properties involved in their hydrolysis. The evidence for this, however, is still rather weak and no detailed study of this problem was made.

(4) All other substrates, ethylbutyrate most probably excepted, were hydrolysed partly by the cholinesterase and partly by the ali-esterase. There is some evidence that *o*-nitrophenylacetate-hydrolysis is partly due to an organo-phosphate-resistant enzyme distinct from the two esterases mentioned above, which are both inhibited by low concentrations of DDVP and paraoxon.

Acknowledgements—The author wishes to thank Mr. C. H. JANSSEN who did some of the experiments dealt with in this paper, and Mrs. I. VAN DER LINDEN-VAN ESCH for her excellent technical assistance. He is especially indebted to Dr. K. A. LORD (Harpenden, G.B.) for much valuable discussion and his willingness to correct the English. Thanks are further due to Prof. Dr. B. MENDEL (Amsterdam) for a gift of butyrylcholine perchlorate, to the Montrose Chemical Company (U.S.A.) for a gift of DDVP and to Prof. J. A. A. KETELAAR (Amsterdam) and to Philips-Roxane Ltd. (Weesp-Holland) for supplying paraoxon.

REFERENCES

ASPEREN K. VAN (1957) Mode of action and metabolism of some organic phosphorus insecticides in houseflies. *Proc. 4th int. Congr. Crop Protection, Hamburg* 1957. In press.

ASPEREN K. VAN (1958) Mode of action of organophosphorus insecticides. *Nature, Lond.* **181**, 355–356.

ASPEREN K. VAN (1958a) The mode of action of an organophosphorus insecticide (DDVP). *Ent. exp. appl.* **1**, 130–137.

ASPEREN K VAN and DEKHUIJZEN H. M. (1958) A quantitative analysis of the kinetics of cholinesterase inhibition in tissue homogenates of mice and houseflies. *Biochim. biophys. Acta* **28**, 603–613.

AUGUSTINSSON K. B. (1950) Acetylcholine esterase and cholinesterase, in *The Enzymes* **1** (1), 443–472. Academic Press Inc. New York.

BERGMANN F. and SHIMONI A. (1953) The enzymic hydrolysis of alkyl fluoroacetates and related compounds. *Biochem. J.* **55**, 50–57.

CHADWICK L. and HILL D. (1947) Inhibition of cholinesterase by di*iso*propyl fluorophosphate, physostigmine and hexaethyl tetraphosphate in the roach. *J. Neurophysiol.* **10**, 235–246.

CHADWICK L. E., LOVELL J. B., and EGNER V. E. (1953) The effect of various suspension media on the activity of cholinesterase from flies. *Biol. Bull., Woods Hole* **104**, 323–333.

CHEFURKA W. and SMALLMAN B. N. (1956) The occurrence of acetylcholine in the housefly, *Musca domestica* L. *Canad. J. Bioch. Physiol.* **34**, 731–742.

COLHOUN E. H. (1958) Tetraethyl pyrophosphate and acetylcholine in *Periplaneta americana*. *Science* **127**, 25.

GOLDSTEIN A. (1954) Substrate summation experiments with cholinesterases. *Fed. Proc.* **13**, 358–359.

HESTRIN S. (1949) The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine, and its analytical application. *J. biol. Chem.* **180**, 249–261.

HOPF H. S. (1952) Injection experiments on the role of cholinesterase inhibition. *Ann. appl. Biol.* **39**, 193–202.

HOPF H. S. (1954) Inhibition of the acetylesterases of the locust nerve cord by some organic phosphoric esters. *Ann. appl. Biol.* **41**, 248–260.

LEHMANN H. and SILK E. (1955) Protection of rabbits against benzoylcholine by injection of pseudocholinesterase. *Bioch. J.* **59**, vii.

LEWIS S. E. (1953) Acetylcholine in blowflies. *Nature, Lond.* **172**, 1004.

LEWIS S. E. and FOWLER K. S. (1956) Effect of di*iso*propyl phosphorofluoridate on the acetylcholine content of flies. *Nature, Lond.* **178**, 919–920.

VOL. 3 1959

LEWIS S. E. and SMALLMAN B. N. (1956) The estimation of acetylcholine in insects. *J. Physiol.* **134**, 241–256.

LORD K. A. and POTTER C (1950) Mechanism of action of organophosphorus compounds as insecticides. *Nature, Lond.* **166**, 893.

LORD K. A. and POTTER C. (1951) Studies on the mechanism of insecticidal action of organophosphorus compounds with particular reference to their anti-esterase activity. *Ann. appl. Biol.* **38**, 495–507.

LORD K. A. and POTTER C. (1954) Differences in esterases from insect species: toxicity of organophosphorus compounds and *in vitro* anti-esterase activity. *J. Sci. Fd. Agric.* **5**, 490–498.

METCALF R. L. (1955) *Organic insecticides.* Interscience Inc. New York, London.

METCALF R. L., FUKUTO T. R., and MARCH R. B. (1958) Mechanisms of action of anticholinesterase insecticides. *Proc. 10th int. Congr. Ent.* **2**, 13–18.

METCALF R. L., MARCH R. B., and MAXON M. G. (1955) Substrate preferences of insect cholinesterases. *Ann. ent. Soc. Amer.* **48**, 222–228.

METCALF R. L., MAXON M., FUKUTO T. R., and MARCH R. B. (1956) Aromatic esterase in insects. *Ann. ent. Soc. Amer.* **49**, 274–279.

POTTER C., LORD K. A., KENTEN J., SALKELD E. H., and HOLBROOK D. V. (1957) Embryonic development and esterase activity of eggs of *Pieris brassicae* in relation to TEPP-poisoning. *Ann. appl. Biol.* **45**, 361–375.

SMALLMAN B. N. (1956) Mechanisms of acetylcholine synthesis in the blowfly. *J. Physiol.* **132**, 343–357.

SMALLMAN B. N. (1958) The physiological basis for the mode of action of organophosphorus insecticides. *Proc. 10th int. Congr. Ent.* **2**, 5–12.

SMALLMAN B. N. and FISCHER R. W. (1958) The effect of anticholinesterases on acetylcholine levels in insects. *Proc. 10th int. Congr. Ent.* **2**, 225–226.

WOLFE L. S. and SMALLMAN B. N. (1956) The properties of cholinesterase from insects. *J. cell. comp. Physiol.* **48**, 215–235.

VOL.
3
1959

PAPERS TO BE PUBLISHED IN FUTURE ISSUES

W. F. BALDWIN and T. N. SALTHOUSE: Dermal glands and mucin in the moulting cycle of *Rhodnius prolixus* Stål.

J. P. HESLOP and J. W. RAY: The reaction of the cockroach *Periplaneta americana* L. to bodily stress and DDT

E. N. LAMBREMONT: A study of acid phosphatase of the mosquito *Aedes aegypti* (L.)

C. B. CARRINGTON and S. M. TENNEY: Chemical constituents of haemolymph and tissue in *Telea polyphemus* Cram.—With particular reference to the question of ion binding

G. HOYLE: The neuromuscular mechanisms of an insect spiracular muscle

B. JOHNSON: Studies on the degeneration of the flight muscles of alate aphids —II. Histology and control of muscle breakdown

B. BHEEMESWAR and P. FAULKNER: Metabolism of oximes in the silkworm *Bombyx mori* L.—I. Inhibition of transaminases

F. MORIARTY: The 24-hour rhythm of emergence of *Ephestia kühniella* Zell. from the pupa

P. S. CHEN: Studies on the protein metabolism of *Culex pipiens* L.—III. A comparative analysis of the protein contents in the larval haemolymph of autogenous and anautogenous forms

P. FAULKNER and B. BHEEMESWAR: Metabolism of oximes in the silkworm *Bombyx mori* L.—II. Metabolism of pyruvic oxime $-2\,^{14}C$.

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 325 *to* 334. *Pergamon Press Ltd., London. Printed in Great Britain*

A STUDY OF ACID PHOSPHATASE OF THE MOSQUITO *AEDES AEGYPTI* L.*

EDWARD N. LAMBREMONT

Department of Zoology and Entomology, The Ohio State University, Columbus†

(*Received* 17 *November* 1958)

Abstract—Acid phosphatase has been studied in total homogenates of adult females of *Aedes aegypti* L. Acid phosphatase when studied on disodium β-glycerophosphate possesses the following characteristics: soluble in water; confined to the supernatant fraction of centrifuged homogenates; kinetics zero order; pH optimum 4·6–5·3; temperature optimum 45°C; Q_{10} 2·13 (25–35°C); activated by Mg (optimum 0·08–0·16 M); Ca, Co, and Fe; inhibited by Mg (0·30 M or higher), fluoride, azide, and oxalate.

VOL.
3
1959

INTRODUCTION

THE need for more detailed studies of the physiology of senescence has been emphasized by several of the better known contemporary gerontologists and prompted the writer to undertake a study of aging in adults of the yellow fever mosquito, *Aedes aegypti* L. Since the enzyme acid phosphatase is known to play important roles in intermediary metabolism, it was planned to study the levels of activity of this enzyme in adults of different ages as a possible indication of physiological aging. Although several papers had been published by other workers studying alkaline phosphatases, it was apparent early in the investigation that very little information was available concerning acid phosphatase in insects, and it was decided to postpone the aging studies until a biochemical characterization could be completed. This paper presents the findings of the biochemical studies of acid phosphatase as it reacts upon disodium β-glycerophosphate in total homogenates of adults of this species. The results of studies of physiological aging are to be presented elsewhere.

MATERIALS AND METHODS

The methods used for obtaining a large number of homogenous adults of this species have been outlined by BURCHAM *et al.* (1956) and were used for the present study.

From preliminary experiments it was determined that the peak of acid phosphatase activity occurred within 24 hr after emergence. A cage containing an emergence tray of approximately 1200 pupae, all of which had been reared at the

* Part of a dissertation presented to the Graduate School of The Ohio State University in partial fulfilment of the requirements for the degree Doctor of Philosophy.

† Present address: United States Department of Agriculture, ARS, Entomology Research Division, Agricultural Center, University Station, Baton Rouge, Louisiana.

same time and under uniform laboratory conditions (80°F, 45 per cent relative humidity), was maintained in the rearing room, and the tray was removed every 24 hr and transferred to an empty cage. The emerged adults were maintained on 10 per cent sucrose solution. The mosquitoes which had emerged were killed by placing the entire cage in the deep freezer at — 20°C, which resulted in almost immediate inactivation, with freezing following within several minutes. The frozen adults were stored in the deep freezer until used.

By preliminary experiments it was determined that a homogenate containing the equivalent of 16 mosquitoes per millilitre would be sufficiently concentrated to release inorganic phosphate in amounts easily detectable by the quantitative capabilities of the equipment available. Hence, that concentration was maintained except for those experiments dealing with the effects of homogenate concentration itself. Only adult females were used. No attempt was made to select only perfect specimens, or to use only the larger ones, and it was assumed that all the mosquitoes taken from the cage had actually died in the freezing process. Since there was a very small amount of tissue in the legs, the absence of appendages was not cause to reject a specimen. GONDA *et al.* (1957), working with particulate fractions of *Aedes aegypti*, concluded that the largest portion of all oxidative enzyme activity was located in the thorax, presumably in the flight muscles. They decided that the large numbers of mosquitoes necessary for the preparation of enzyme source made the isolation of thoraces impracticable. Although it is often difficult to make absolute determinations about enzymes being studied in total homogenates, or to characterize them on the basis of only one or two co-factors, the convenience of employing the homogenate technique with such small insects was deemed advisable.

The frozen adults were homogenized in a Teflon-pyrex tissue grinder in an ice bath, with a small amount of ice-cold, demineralized water. Grinding was continued for exactly 3 min. The homogenates were then filtered through a Gooch crucible fitted exactly with a disk of uniform weight, thickness, and diameter, cut from a large sheet of compressed fibre glass. The pestle and grinding tube were washed with a small volume of cold, demineralized water, and this was added to the filtering disk to obtain the desired volume. Filtered homogenates were returned to the freezer until time for use.

Acid phosphatase was assayed using a slight modification of the method of ROCKSTEIN and HERRON (1951a) and ROCKSTEIN (1956a, 1956b) by combining the following reagents: 0·75 ml of 0·8 M $MgCl_2$, 0·90 ml of 0·33 N acetic acid, 0·60 ml of molar Na_2 β-glycerophosphate prepared in 0·848 per cent sodium barbital, and two small drops of chloroform. This mixture will be referred to as the basic reaction mixture. The pH of the reaction mixture was 5·4. One and one-half millilitre of freshly thawed cold homogenate was pipetted into the mixture, which was agitated vigorously for a few seconds. Immediately, 1·25 ml of the total reaction mixture was removed and added to a tube containing 0·25 ml of 30 per cent trichloroacetic acid (TCA) as a zero time control. Incubation was conducted for exactly 30 min (plus or minus 15 sec) in a water bath at 35°C. After incubation, 0·50 ml of TCA was added to the reaction tube, and the zero

time and activity tube contents were filtered through Whatman No. 42 paper. Aliquots (0·2 ml) of the deproteinized filtrates were used to determine the zero time and the enzymatically-released orthophosphate.

Assay of released orthophosphate followed ROCKSTEIN and HERRON (1951b). Activity is expressed as net increase (activity minus zero time control) in optical density (O.D.) at 720 mμ of 0·2 ml of final deproteinized reaction mixture.

RESULTS AND DISCUSSION OF BIOCHEMICAL CHARACTERIZATIONS

Location of activity in fractions of centrifuged homogenates. Six millilitres of homogenate were pipetted into a chilled centrifuge tube and spun at 3000 g in a Sorvall angle-head centrifuge at 1–3°C. The supernatant fraction was decanted and retained for later use. The solid fraction was dispersed in 6 ml of cold water, again sedimented by centrifuging, resuspended in 6 ml of cold water, and retained for further analysis. Aliquots of the first supernatant, and washed and resuspended particulate fractions, were used in assaying for enzyme activity according to the basic reaction mixture.

The results clearly indicated that the activity was in the supernatant or soluble fraction, and a trace amount of activity found in the solid fraction was attributed to insufficient washing. Thus, as far as solubility is concerned, the acid phosphatase of *Aedes aegypti* is identical with that of the classical vertebrate enzyme.

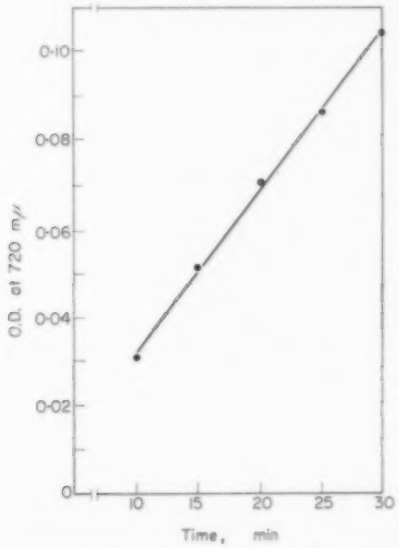


FIG. 1. Kinetics of acid phosphatase.

Kinetics of enzyme action. At intervals of 10, 15, 20, 25, and 30 min aliquots were removed from the basic reaction mixture and assayed for orthophosphate released in relation to duration of incubation.

The results are presented in Fig. 1 and clearly indicate that the reaction was of the zero order. These results are similar to those of FITZGERALD (1949) for alkaline phosphatase in the eggs of the grasshopper *Melanoplus differentialis*, and to results obtained by ROCKSTEIN (1956a) who used total homogenates of housefly adults, and to honey bee acid phosphatase data reported by ROCKSTEIN and HERRON (1951a). ROCKSTEIN (1956a) found that a zero order reaction was maintained for approximately 40 min by alkaline phosphatase, after which time the reaction began approaching the first order. According to FITZGERALD (1949), the zero order reaction was maintained for more than 1 hr in eggs of *M. differentialis*.

Effect of varying enzyme concentration. Homogenates of the following strengths were prepared: 24, 12, 8, and 4 adult female *Aedes* per millilitre. These were used in basic reaction mixtures from which aliquots were removed at 0, 10, 15, 20, 25, and 30 min, and analysed for released orthophosphate.

A zero order reaction was still retained, even in the presence of a higher enzyme concentration (24 females per millilitre) than was used for the first kinetics studies, although less orthophosphate was split per unit of time by the diluted homogenates. These results are illustrated in Fig. 2.

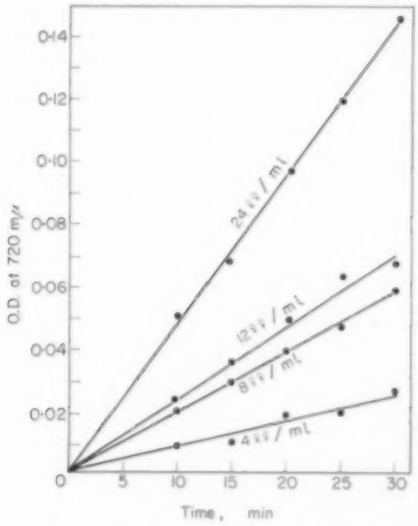


FIG. 2. Effect of varying homogenate concentration on rate of acid phosphatase activity.

Effect of omission of substrate and of varying substrate concentration. Dilutions of Na_2 β-glycerophosphate were prepared with 0·848 per cent sodium barbital to give the following final molarities: 0·24, 0·16, 0·08, 0·032, and 0·016. In addition, a reaction mixture in which substrate was replaced with an equal amount of demineralized water was prepared. Acid phosphatase was determined using these substrate variations according to the basic reaction plan.

Figure 3 illustrates the effect of increasing substrate concentration on enzyme activity; release of orthophosphate increases rapidly as substrate is increased up to about 0·08 M. Similar results were obtained by FITZGERALD (1949) and ROCKSTEIN (1956a) for alkaline phosphatase, and the point at which the *Aedes* graph begins to curve is comparable to the alkaline phosphatase studied by the latter. The Michaelis constant (Km) for acid phosphatase, derived from reciprocal plot according to the method of LINEWEAVER and BURK (1934), was $8{\cdot}57 \times 10^{-2}$ M when determined for the stated reaction conditions.

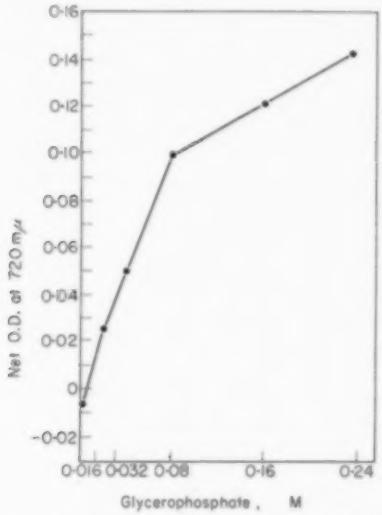


FIG. 3. Effect of substrate concentration on velocity of acid phosphatase.

It is interesting to note that in the tube from which substrate was omitted, a slight uptake of phosphate from the total reaction mixture took place (from activity in the tissue homogenate), since the readings of this tube were less than that of its respective zero time samples. Therefore, a phosphorylating system was operating in the homogenate, and it must be realized that all readings in this study were a measure of a net increase of orthophosphate.

Effect of varying pH. The pH variations for determining a pH curve of acid phosphatase activity were obtained by changing the volume and normality of the acetic acid component while holding the sodium barbital-substrate mixture constant in a manner similar to that of ROCKSTEIN (1956a).

From preliminary experiments it was realized that the pH curve would not have a sharp optimum point of activity, but instead there would be a broad range of near optimal hydrogen ion concentration. Therefore, it became necessary to make repeated determinations in order to clarify points along the pH curve. The pH curve (Fig. 4) for acid phosphatase is, then, a composite approximation of the effects of pH factor.

Figure 4 provides evidence that the range of optimal activity lies between 4·6 and 5·3, which is slightly more acid than expected. However, since all other experiments were run at the (originally) expected peak of 5·4, it was decided to continue the study at that level, as its distance away from the optimal range was not great. This would obviate the necessity of redetermining many of the other factors which had already been studied at pH 5·4.

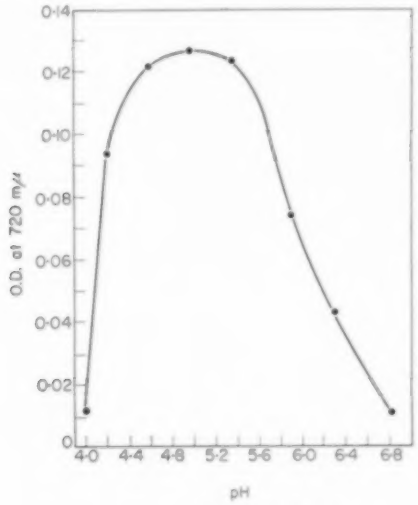


FIG. 4. pH curve for acid phosphatase.

Comparison with the phosphomonoesterases as classified by ROCHE (1951) shows that the acid phosphomonoesterases II and III possess pH ranges of 5·0–5·5 and 3·4–4·2, respectively, although the magnesium ion responses of the two enzymes do not compare with those determined for the *Aedes* acid phosphatase. Phosphomonoesterase IV of Roche, which reacts toward Mg in the same manner as the *Aedes* acid phosphatase, possesses a slightly higher pH range, 5·0–6·0, and it would seem that, if a classification can be made, the isodynamic mixture of acid phosphatase in *Aedes* closely resembles phosphomonoesterase IV. Acid phosphatase as determined by BARKER and ALEXANDER (1958) for *Musca domestica* was studied at pH 4·8, although the effects of other pH levels were not tested. ROCKSTEIN and HERRON (1951a), using total homogenates of worker honey bees, found the pH optimum of the acid enzyme to be 5·6, and judging by a similar activation by Mg ions, these workers also classified the honey bee enzyme as phosphomonoesterase IV.

Effect of varying temperature on enzyme activity; temperature optimum of acid phosphatase. Water baths were standardized to obtain temperatures of 15, 25, 30, 35, 40, 45, 50, and 55°C to a tolerance of 0·5°C. Analysis followed the basic reaction mixture.

The effects of the temperatures studied are presented in Fig. 5, and a summary of the Q_{10} calculations is presented in Table 1. The apparent optimum is near 45°C, with activity falling very sharply at 50°C.

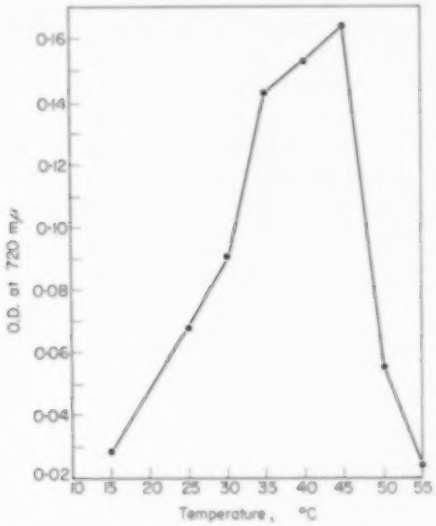


FIG. 5. Effect of temperature on acid phosphatase.

TABLE 1. TEMPERATURE COEFFICIENTS (Q_{10}) FOR *Aedes aegypti* ACID PHOSPHATASE

Temperature range (°C)	Q_{10}
15–25	2·23
25–35	2·13
30–40	1·78
35–45	1·15

BODANSKY (1939) reported that the temperature optimum of mammalian bone phosphatase was from 40 to 42°C. FITZGERALD (1949) found the optimal conditions for alkaline phosphatase of *Melanoplus differentialis* eggs to be at 35°C. ROCKSTEIN and HERRON (1951a) determined that the temperature optimum of alkaline phosphatase of *Apis mellifera* was at 35°C, and that the Q_{10} values range from 2·38 (5–15°C) to 1·78 (15–25°C), and 1·38 (25–35°C). The time of incubation for those determinations was 5 hr. ROCKSTEIN (1956a), studying the alkaline enzyme in *Musca domestica* adults, determined the optimum for that insect to be at 40°C for a 25 min period of incubation.

Effect of magnesium as the activating ion. Aqueous solutions of $MgCl_2$ were prepared to give the following final molarities: 0·0 (demineralized water); 0·01;

0·02; 0·04; 0·08; 0·16; 0·30. These solutions were substituted for the magnesium component in the basic reaction mixture.

A graph of Mg ion effects is presented in Fig. 6, and it is clear that activity is sharply influenced by this cation. In the tube from which Mg was omitted (no Mg other than that originally present in the tissues), activity was approximately a net O.D. of 0·075. As Mg ions were added, activity rose sharply to an optimum of not less than 0·08 M, but definitely less than 0·16 M final concentration. The true optimum probably lies somewhere closer to the lower end of this range. An inhibitory effect resulted in the tube containing the highest molarity (0·30 M). FITZGERALD (1949) found optimal Mg ion concentration at 0·05 M for *Melanoplus* eggs, and ROCKSTEIN and HERRON (1951a) determined the Mg ion activation of acid phosphatase of worker bees to be a final molarity of 0·01.

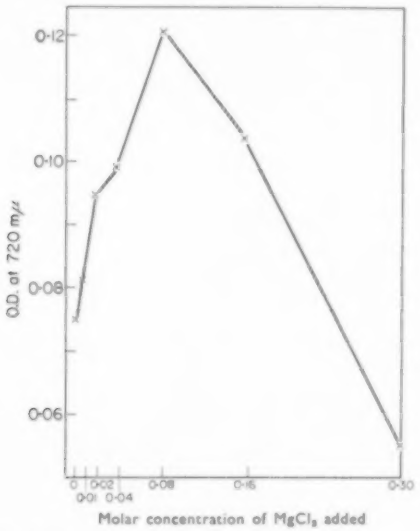


FIG. 6. Effect of Mg ions on acid phosphatase activity.

Effect of the divalent cations Ca, Mn, Co, *and* Fe. Aqueous solutions of the dichloride salts of Ca, Mn, Co, and Fe were prepared to give a 0·16 M final concentration. The Fe solutions were prepared just prior to use. Assay for the release of orthophosphate followed the basic reaction mixture. The pH of the final reaction mixture was checked after incubation to assure that the apparent effects of added ions were not due to a shift of hydrogen ion concentration.

Two series of tests were run, as are indicated in Table 2, which summarizes the effects of cation activators. All of the ions tested activated this enzyme above that in which no extra ions were added; Fe ions brought about the highest activation, Co the lowest when compared with the reaction tube in which water replaced metal activator. The relative degrees of activation by Mn and Ca were difficult to evaluate from these data, since Ca was higher than Mn in Series 1, but lower in

Series 2. Mg ions, as in previous tests, activated strongly. The alkaline phosphatase of grasshopper eggs was activated by both Mg (optimal at 0·05 M) and Ca (optimal at 0·03 M), as reported by FITZGERALD (1949). ROCKSTEIN (1956a) found that the order of activation of housefly alkaline phosphatase increased from a net absorbence of 0·018 (Ca); 0·084 (Mn); 0·085 (Co); 0·125 (Mg); and 0·126 (Fe), and these compare favourably with the acid phosphatase of *Aedes*, although no information was given as to the relative position of a water-substituted mixture.

TABLE 2. EFFECTS OF PRESENCE OR ABSENCE OF DIVALENT CATIONS ON ACID PHOSPHATASE ACTIVITY (ACTIVITY EXPRESSED AS NET OPTICAL DENSITY OF 0·2 ML OF FINAL REACTION MIXTURE; pH OF FINAL MIXTURE IN PARENTHESES)

Series	H_2O	Mg	Mn	Ca	Fe	Co
1	·063	—	0·094	0·104	0·127	0·078
pH	(6·15)	—	(5·32)	(5·70)	(5·07)	(5·45)
2	0·044	0·121	0·094	0·084	—	—
pH	(6.13)	(5·42)	(5·33)	(5·72)	—	—

VOL. 3 1959

Effects of inhibitors on enzyme activity. The reaction mixture for studying inhibitors differed slightly from the original reaction plan in that 0·5 ml of appropriate inhibitor or water blank and 0·75 ml of demineralized water were added to the basic reaction mixture. The pH of the final mixtures was taken after incubation to insure the effects had been due to inhibitor and not to pH shift.

The following inhibitors were tested at the indicated final molar concentrations: sodium fluoride (0·005 M); sodium azide (0·01 M); sodium oxalate (0·01 M), and water in lieu of inhibitor.

The results clearly indicated that the inhibitors tested depress enzyme activities. Acid phosphatase activity was inhibited 88 per cent by fluoride, 55 per cent by oxalate, and 36 per cent by azide when compared to the reaction mixture in which water replaced inhibitor. There was no significant change in pH. Fluoride is a classic inhibitor of the phosphatases. Phosphomonoesterase II is strongly inhibited by fluoride at low concentrations (0·001–0·005 M), which agrees with the 88 per cent inhibition of the *Aedes* phosphatase of the present study. The same statement may be made concerning oxalates, as sodium oxalate produced a 55 per cent inhibition of the acid phosphatase studied. Likewise, azide inhibition of 36 per cent agrees with previous results reported for alkaline phosphatase (ROCHE, 1951). BRADFIELD (1951), using histochemical methods to study phosphatases of the goat moth larva (*Cossus cossus*) and the silk-worm larva (*Bombyx mori*), and several species of spiders, found that silk gland phosphatases were not affected by 0·001 M Hg, Ag, Cu, Zn, or iodoacetate, or by 0·0001 M *p*-chlormercuribenzoate.

SUMMARY AND CONCLUSIONS

An investigation of the enzyme acid phosphatase of the mosquito *Aedes aegypti* L. resulted in the characterizations summarized as follows: soluble in water; activity located in the supernatant fraction of centrifuged homogenates; kinetics,

zero order; Km $8{\cdot}57 \times 10^{-2}$; pH optimum 4·6–5·3; temperature optimum 45°C; Q_{10} 2·13 between 25–35°C; activated by Mg, Ca, Co, and Fe, inhibited by fluoride, azide and oxalate, and Mg at concentrations above 0·3 M.

Comparison of these results with those reported in the literature for several other species of insects leads to the conclusion that the enzyme is basically the same as similar enzymes of other insect species, and furthermore is comparable to the better known vertebrate enzyme in most respects.

Acknowledgements—The writer wishes to thank Dr. F. W. FISK, The Ohio State University, for encouragement during the research and for accurate editorship. Suggestions and valuable guidance were given by Dr. M. ROCKSTEIN, New York University College of Medicine. Dr. R. BARKER, U.S.D.A., Beltsville, Maryland, willingly sent his data and comments on housefly phosphatases.

Support for the research came from Research Grant E-528(C7), the National Institutes of Health, U.S. Public Health Service. To Dr. D. DELONG, Supervisor of the Mosquito Research Project supported by this grant, and Dr. A. O. LEA, Research Associate, the writer owes thanks.

REFERENCES

BARKER R. J. and ALEXANDER B. H. (1958) Acid and alkaline phosphatase in houseflies of different ages. *Ann. ent. Soc. Amer.* **51**, 255–257.

BODANSKY O. (1939) Energy of activation of the hydrolysis of sodium β-glycerophosphate by bone phosphatase at optimal pH. *J. biol. Chem.* **129**, 197–206.

BRADFIELD J. R. G. (1951) Phosphatases and nucleic acids in silk glands; cytochemical aspects of fibrillar protein secretion. *Quart. J. micr. Sci.* **92**, 87–112.

BURCHAM E. G., LEA A. O., DIMOND J. B., and DELONG D. M. (1956) Laboratory rearing procedures for *Aedes aegypti* and *Culex pipiens*. *North Central Branch Papers, E.S.A. Wis. Alumni Res. Found.* Insecticide Testing Lab., Madison, Wis. 7 pp.

FITZGERALD L. R. (1949) The alkaline phosphatase of the developing grasshopper egg. *J. exp. Zool.* **110**, 461–487.

GONDA O, TRAUB A., and AVI-DOR Y. (1957) The oxidative activity of particulate fractions from mosquitoes. *Biochem. J.* **67**, 487–493.

LINEWEAVER H. and BURK D. (1934) Determination of enzyme dissociation constants. *J. Amer. chem. Soc.* **56**, 658–666.

ROCHE J. (1951) Phosphatases. *The Enzymes* (Ed. by J. B. Sumner and K. Myrbäck). Academic Press, New York. **1**, (1) 473–510.

ROCKSTEIN M. (1956a) Phosphatases of the housefly, *Musca domestica*. *Bull. Brooklyn ent. Soc.* **51**, 8–17.

ROCKSTEIN M. (1956b) Some biochemical aspects of aging in insects. *J. Geront.* **11**, 282–285.

ROCKSTEIN M. and HERRON P. W. (1951a) Phosphatase in the adult worker honey bee. *J. cell. comp. Physiol.* **38**, 451–468.

ROCKSTEIN M. and HERRON P. W. (1951b) Colorimetric determination of inorganic phosphate in microgram quantities. *Analyt. Chem.* **23**, 1500–1501.

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 335 to 344. *Pergamon Press Ltd., London. Printed in Great Britain*

STUDIES ON THE PROTEIN METABOLISM OF *CULEX PIPIENS* L.—III

A COMPARATIVE ANALYSIS OF THE PROTEIN CONTENTS IN THE LARVAL HAEMOLYMPH OF AUTOGENOUS AND ANAUTOGENOUS FORMS*

P. S. CHEN

Institute of Zoology and Comparative Anatomy, University of Zürich, Switzerland

(*Received* 24 *November* 1958)

Abstract—The protein contents in the larval haemolymph of *Culex pipiens* (autogenous form) and *Culex fatigans* (anautogenous form) have been determined by paper electrophoresis. In both mosquito strains the electropherograms showed only one protein fraction which increased rapidly in its concentration during the later period of larval development. The fully grown larva of the anautogenous form was found to contain more proteins per unit volume blood than that of the autogenous form. On the other hand, the total blood concentration of the free ninhydrin-reacting components, as estimated by the method of paper chromatography, is higher in the autogenous form than in the anautogenous form. On the basis of data presented in this study, the relation of nutritional reserves to the phenomenon of autogenous egg production in mosquitoes is discussed.

VOL.
3
1959

INTRODUCTION

IT is known that the common house mosquito, *Culex pipiens* L., includes two forms which differ in their mode of egg formation. The autogenous form is able to lay its first batch of eggs without taking a blood meal or other food from an outside source, whereas the anautogenous form will not lay eggs until blood has been ingested. Numerous investigations have been made on the ecology, taxonomy, and genetics of these two mosquito strains (for literature see LAVEN, 1957). However, we still know very little about the physiology of autogeny.

In anautogenous mosquitoes the substances, especially proteins, contained in the ingested blood evidently provide a large part of the necessary material for yolk formation. It has been suggested that in autogenous mosquitoes sufficient nutritional reserves are stored up by the larva and later used by the adult for ovarian development (BOISSEZON, 1933; ROUBAND, 1933). For instance, larvae of the autogenous type were found to contain more reserves than those of the anautogenous type raised under equal conditions (ROUBAND and TOUMANOFF, 1930; ROUBAND, 1933; MÖLLRING, 1956). At emergence autogenous females were shown to have more fat reserves than anautogenous females (MÖLLRING, 1956; TWOHY and ROZEBOOM, 1957). However, evidence has been brought forward to

* The present work was supported by a grant from the "Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung".

show that the extent of nutritional reserves influenced only the number of eggs laid by the adult and had no effect on the initiation of autogenous ovary development (GASCHEN, 1932; HECHT, 1933). Females, even when reared on a limited amount of food in the larval stage, were always autogenous (BUCK, 1935; MÖLLRING, 1956). Furthermore, examination of the fat body demonstrated that anautogenous mosquitoes contained a sufficient amount of reserves to produce some eggs (CLEMENTS, 1956). From these findings it appears that the quantity of nutritional reserves is not the deciding factor for autogeny or anautogeny.

More recently, evidence has been provided to indicate that the ovarian development in mosquitoes is under hormonal control (DETINOVA, 1945; MEDNIKOVA, 1952; CLEMENTS, 1956; GILLETTE, 1957; LARSEN, 1958). It seems that in autogenous females a gonadotropic hormone is secreted by the corpora allata even without feeding, whereas in anautogenous females the intake of blood is necessary for activation of the hormone system.

Suggestions have also been made that some unknown chemical components in the blood are responsible for anautogenous egg production (YOELI and MER, 1938; GREENBERG, 1951; HOSOI, 1954). For yolk formation the protein reserves are no doubt of primary importance. The present study was carried out to discover if there are qualitative or quantitative differences in blood proteins between larvae of the autogenous form and those of the anautogenous form. The results, reported here, supplement further the previous study dealing with metabolic changes of free amino-acids during the larval development of *Culex pipiens* (CHEN, 1958).

MATERIALS AND METHODS

Materials used in the present study were the autogenous form of *Culex pipiens* L. and the anautogenous form of *Culex fatigans* Wied. Both strains were cultured for many generations first in the Max-Planck Institute of Biology, Tübingen, and later in this laboratory.* The taxonomy of the *Culex pipiens* complex is still a matter of dispute and the consideration of these two strains as different species has been questioned (FARID, 1949; ROZEBOOM, 1951; LAVEN, 1953).

Conditions for rearing the larvae were the same as described previously except that the culture dishes were maintained at 25°C (see CHEN, 1958). During larval development the number of individuals and the amount of food (powdered dog-biscuit) per dish were carefully controlled. The larvae of both strains were raised under identical conditions and only individuals from parallel cultures were used for comparison.

The paper electrophoresis technique was used for the determination of protein contents in the larval haemolymph (for a detailed description of the method, see DURRUM, 1950; WUNDERLY, 1953). Larvae of desired developmental stages were first collected from culture dishes, cleaned thoroughly in distilled water, and dried on filter paper. The specimens were then opened individually under a binocular microscope on a paraffin-coated slide, and the blood was collected in a calibrated

* I wish to thank Dr. H. LAVEN, Tübingen, for supplying the *Culex* strains.

VOL. 3
1959

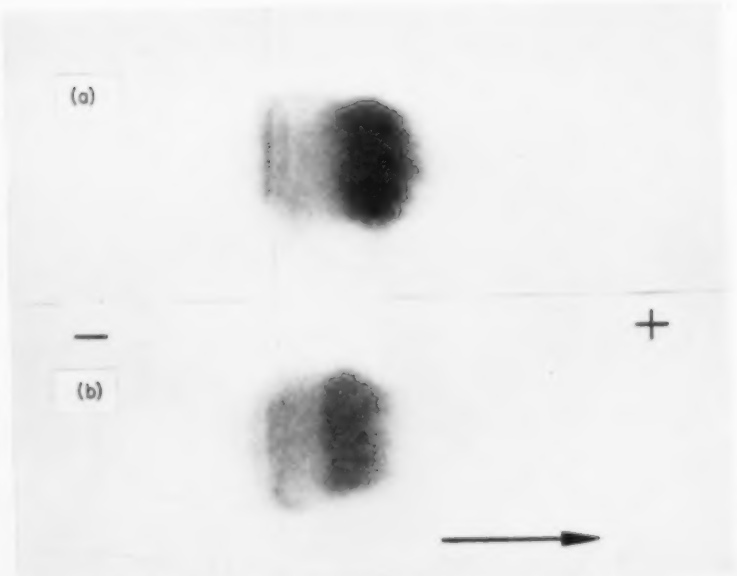


FIG. 1. Protein fractions in the haemolymph (30 μl) of fully grown larvae of *Culex pipiens* (a) and *Culex fatigans* (b).

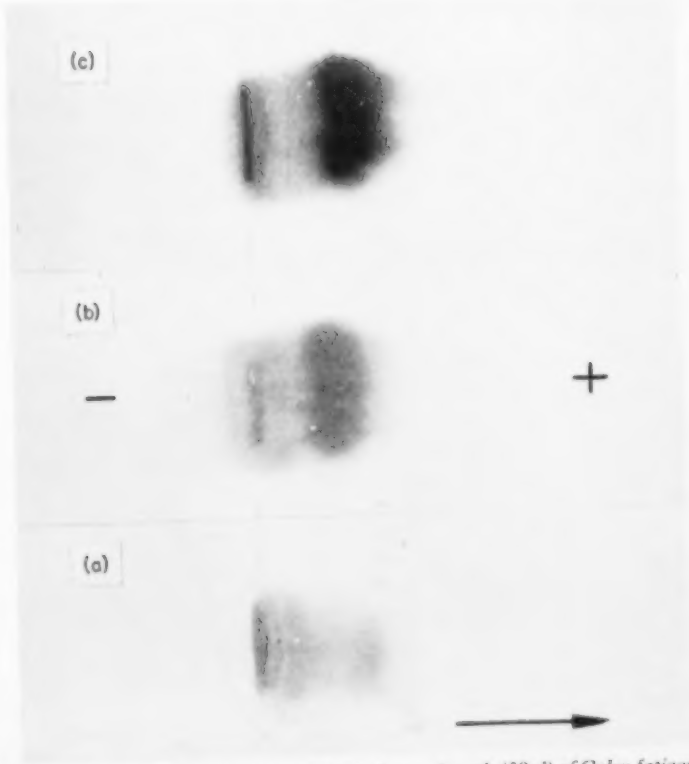


FIG. 2. Changes of protein contents in the haemolymph (30 μl) of *Culex fatigans* larvae aged 8 (a), 10 (b) and 12 (c) days after hatching at 25°C.

micropipette. During dissection care was taken to avoid injury of the internal organs. For each protein determination 20–30 μl of haemolymph was used. It is known that insect blood, when exposed to air, forms a black pigment which disturbs the separation of proteins during electrophoresis (see CHEN, 1956). In order to prevent enzymatic pigment formation, each blood sample was therefore diluted with a saturated solution of phenylthiourea (2 parts of haemolymph to 1 part of phenylthiourea solution), and kept for about 30 min in a desiccator which contained potassium cyanide.

After the above treatments, the blood sample was centrifuged at 4500 rev/min for about 5 min and delivered with a fine pipette as a thin line to the middle of a filter paper sheet (Whatman No. 1, 7 × 40 cm). The separation of the protein fraction was carried out in a buffer solution (veronal-acetate) of pH 8·6 with a current of 175 V and 3·5 mA. After 16 hr the paper strip was stained with 0·1 per cent bromophenol blue in absolute methanol containing 6 per cent $HgCl_2$, washed in running water, and dried at room temperature. For quantitative determination of the protein fraction the stained sheet was cut into 5 mm wide strips perpendicular to the separation direction, and each strip eluted in a test-tube containing 5 ml of 50 per cent alkaline methanol. The colour intensity of the methanol solution was estimated spectrophotometrically at a wave-length of 595 mμ. Finally, the extinction values were plotted on a graph sheet and the area between the plotted curve and the base line was measured by a planimeter.

For the analysis of amino-acid components, the protein fraction was eluted from the paper and hydrolysed in 6 N HCl at 110°C for 12 hr. The hydrolysate was neutralized with 0·1 N NaOH and applied again to a Whatman No. 1 sheet (28·6 × 46·2 cm) for two-dimensional paper-chromatographic separation.

In addition to the protein contents the free ninhydrin-reacting substances in the larval haemolymph of both strains were also compared. The method used was the same as that described in a previous paper (CHEN, 1958). However, the colour reaction was done by spraying the sheet with 0·5 per cent ninhydrin in 96 per cent ethanol, followed by an acid $Cu(NO_3)_2$ solution (2 ml saturated aqueous Cu $(NO_3)_2$ + 0·2 ml 10 per cent HNO_3 + 200 ml 96 per cent ethanol). The spots were then extracted in 5 ml absolute methanol and the colour intensity evaluated at a wave-length of 510 mμ (for a detailed description of the technique, see BODE *et al.*, 1952; FISCHER and DÖRFEL, 1953; BENZ, 1957).

RESULTS

1. *Comparison of protein contents in the larval haemolymph*

By the paper-electrophoresis technique used in the present study only one protein fraction could be detected in the haemolymph of *Culex* larvae (Fig. 1). Very recently, using the technique of starch-gel-electrophoresis, it was found that the blood proteins of the *Culex* larvae can be separated into at least three to four fractions (CHEN, 1959). This shows that the protein fraction obtained by paper electrophoresis is by no means homogeneous. This is in contrast to *Drosophila* which has been shown to contain at least two protein fractions during the later

part of larval development (WUNDERLY and GLOOR, 1953; CHEN, 1956). As shown in Figs. 2 and 3, the protein contents in the larvae of both *C. pipiens* and *C. fatigans* increase with age. The blood concentration is very low until the eighth day after hatching and increases about 4 times in the next 4–6 days (Table 1). The values rise from 0·188 to 0·766 unit per 20 μl haemolymph for *C. pipiens* and 0·259 to 1·072 for *C. fatigans*. This fact is in agreement with the earlier finding that both fresh weight and total N of *Culex* larvae increase rapidly at the same developmental period (cf. Fig. 1 in CHEN, 1958, and Fig. 4 in the present study).

It is of interest to notice that the protein concentration in the larvae of the anautogenous form (*C. fatigans*) is higher than that in the larvae of the autogenous form (*C. pipiens*) of corresponding developmental ages (Figs. 1 and 3). Quantitative

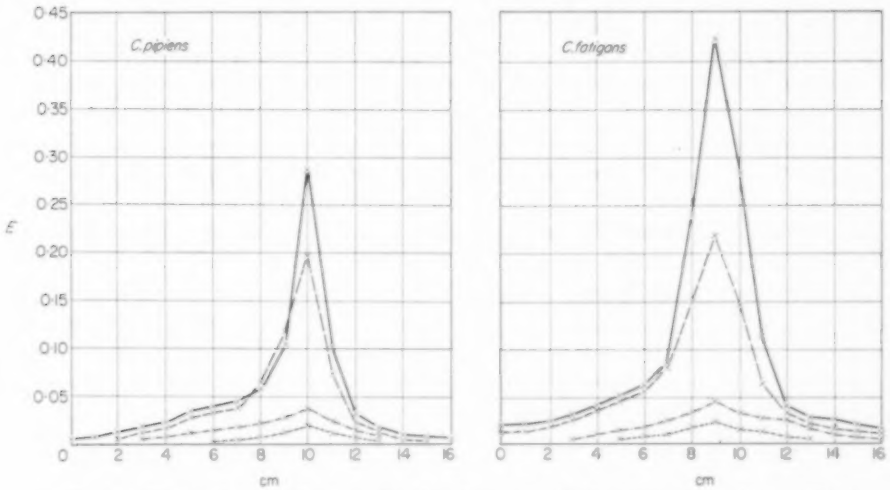


FIG. 3. Electrophoretic diagrams showing the successive increase of protein contents in the haemolymph of *Culex fatigans* and *Culex pipiens* larvae at 8 (- - - -), 10 (—·—), 12 (— — —) and 14 (—) days of age at 25°C. Ordinate: extinction (E) per 20 μl haemolymph at 595 mμ.

TABLE 1. CHANGES OF PROTEIN CONCENTRATIONS IN THE HAEMOLYMPH OF *Culex pipiens* AND *Culex fatigans* DURING LARVAL DEVELOPMENT

Larval age (days after hatching at 25°C)	Concentration of proteins (extinction per 20 μl haemolymph)			
	Culex pipiens		*Culex fatigans*	
	n	M ± S.E.	n	M ± S.E.
8–9	5	0·188 ± 0·0358	5	0·259 ± 0·0253
10–11	4	0·351 ± 0·0136	4	0·388 ± 0·0595
12–14	11	0·766 ± 0·0441	9	1·072 ± 0·0742

n= number of determinations.

determinations showed that this difference could be detected at all three larval stages investigated in the present study (Table 1). However, the difference is significant only for individuals at 12–14 days of age (t-test, $p < 0.01$).

2. *Changes of fresh weights during larval development*

In view of the fact that protein synthesis is especially intensive in the later part of larval life, and that in the above experiment the absolute age, i.e. the age determined from hatching, was used as a basis for comparison, the divergence in protein contents just described could be due to a difference in developmental rates between the two mosquito strains. MÖLLRING (1956) observed that the larval life of the autogenous form lasted 2½ days longer than that of the anautogenous form under the same condition. Similar observations have been reported by TWOHY and ROZEBOOM (1957). Thus, an analysis of the growth of both *C. pipiens* and *C. fatigans* larvae was carried out. The changes in fresh weights of individuals from 42 cultures of 4 parallel series were determined. As illustrated in Fig. 4 the increase of body weights is the same in both strains during the first six days of larval life. From the eighth day onwards, the values of *C. fatigans* always remain somewhat lower than those of *C. pipiens*. According to observations of five parallel cultures started at the same time and kept under identical conditions, the pupation of *C. fatigans* larvae began on the average 24 hr earlier than that of *C. pipiens* (Fig. 4). The average duration of larval development per culture

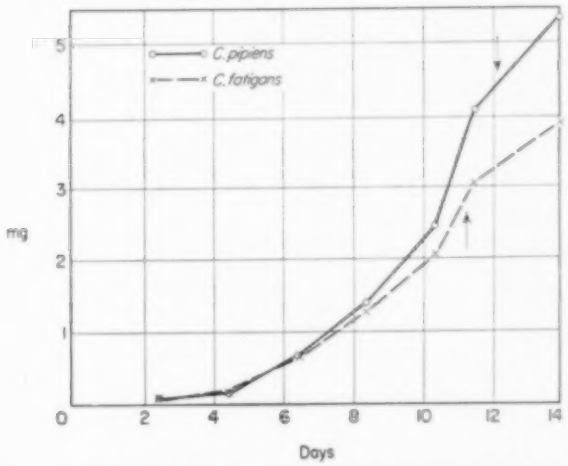


FIG. 4. Changes in fresh weights during larval development of *Culex pipiens* (—) and *Culex fatigans* (— — —). Ordinate: fresh weight in mg per larva. Abscissa: larval age in days after hatching at 25°C. Each point represents the average value of 5–16 determinations. The arrows indicate the beginning of pupation.

dish at 25°C was estimated to be 12–15 days for *C. fatigans* and 13–18 days for *C. pipiens*. This result seems to suggest that the differences in protein contents listed in Table 1 were really due to a higher developmental rate in the anautogenous

form than in the autogenous form. However, determinations carried out on *C. pipiens* larvae 15 and 17 days old showed no significant further increase in their protein contents, and the values at such later stages remained lower than the maximal values estimated for *C. fatigans* larvae of corresponding physiological age. In other words, even if the difference in the developmental rate is considered, the blood proteins still appear more concentrated in the anautogenous form than in the autogenous form.

3. *Comparison of free ninhydrin-reacting substances in the larval haemolymph*

Since the two *Culex* strains differ in their protein contents, the question arises whether there are also differences in free amino-acids and related substances. Investigations of the blood of fully grown larvae showed that the patterns of ninhydrin-reacting components in both forms were the same; they consisted of at least sixteen amino-acids, glutamine and three peptides as reported previously (cf. Fig. 2 in CHEN, 1958). Quantitative determinations of the total concentration in larvae 8–14 days old revealed the following facts (Table 2): (1) in both strains the values increased slightly at first and remained largely unchanged during later development; (2) *C. pipiens* showed consistently higher values than *C. fatigans*, although again only the difference at the last larval stage examined is significant ($p < 0.001$).

4. *Characterization of the protein fraction*

From previous experience it is known that the amount of dye bound per unit weight of protein varies from protein to protein. CREMER and TISELIUS (1950) found that the amount of bromophenol blue taken by serum albumins was about

TABLE 2. TOTAL CONCENTRATIONS OF FREE NINHYDRIN-REACTING SUBSTANCES IN THE HAEMOLYMPH OF *Culex pipiens* AND *Culex fatigans* DURING LARVAL DEVELOPMENT

Larval age (days after hatching at 25°C)	Total concentration of free ninhydrin-reacting substances (extinction per 2 µl haemolymph)			
	Culex pipiens		*Culex fatigans*	
	n	M ± S.E.	n	M ± S.E.
8–9	16	0·492 ± 0·0265	13	0·439 ± 0·0299
10–11	7	0·631 ± 0·0278	6	0·564 ± 0·0169
12–14	17	0·612 ± 0·0123	17	0·526 ± 0·0159

n= number of determinations.

1·6 times as great as that taken by an equal weight of serum globulins. For quantitative work it is necessary to have more information about the nature of the proteins. We know almost nothing about the nature of blood proteins in insects. Therefore, efforts have been made to characterize the protein fraction separated in this study.

VOL.
3
1959

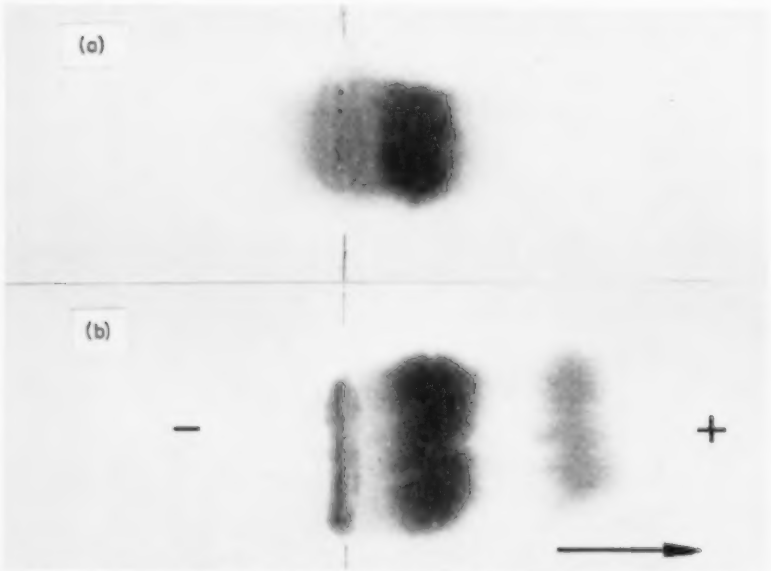


FIG. 5. Comparison of electrophoretic patterns between fully grown *Drosophila melanogaster* (a) and *Culex fatigans* (b) larvae. Protein fractions in 25 μl haemolymph.

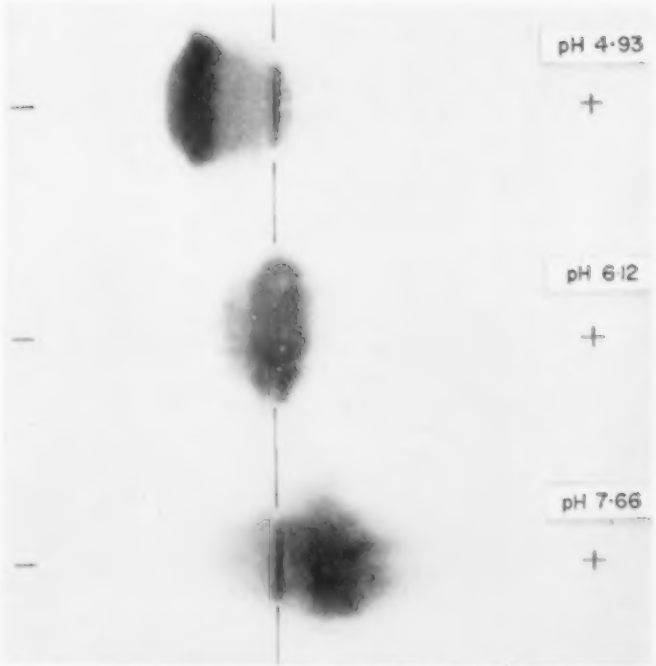


FIG. 6. Determination of the isoelectric point of the larval blood proteins of *Culex pipiens* at pH 4·93–7·66.

The acid hydrolysis indicated that the protein fractions of both *C. pipiens* and *C. fatigans* contained the same amino-acid components. At least the following amino-acids have been identified in the hydrolysates: aspartic acid, glutamic acid, serine, glycine, threonine, tyrosine, α-alanine, β-alanine, lysine, arginine, valine (and/or methionine), proline, leucine (and/or isoleucine). Quantitative estimations detected no difference in the relative amount of each substance in both forms.

Judging from its mobility, the protein fraction of *Culex* corresponds apparently to the so-called B-fraction of *Drosophila* (WUNDERLY and GLOOR, 1953; CHEN, 1956). The distances travelled by the blood proteins of both insects on the paper electropherograms are illustrated in Fig. 5. The less concentrated A-fraction of *Drosophila* larva could not be detected in *Culex* even in individuals which were ready for pupation.

The determination of the isoelectric point showed that at pH 7·66 the protein fraction still moved in the anode direction (Fig. 6). It travelled towards the cathode at pH 4·93. In a buffer of pH 6·12 no movement was observed. In this respect the blood proteins of *Culex* larva are more similar to serum globulins (isoelectric point = 5–7) and differ distinctly from serum albumin which has an isoelectric point of pH 4·2.

VOL. 3 1959

DISCUSSION

Several studies on the blood proteins of insects by the electrophoresis technique have been made. CLARK and BALL (1956) found in the homogenates of *C. pipiens* and *C. stigmatosoma* four protein fractions, and in the haemolymph of *Estigmene acrea*, *Papilio zelicaon*, *Protoparce sexta*, and *Periplaneta americana* four to six protein components. KRIEG (1956) found in the larval haemolymph of *Melolontha* sp., *Neodiprion sertifer*, and *Lymantria dispar* at least two protein fractions. By the method of starch electrophoresis DENUCÉ (1957, 1958) observed a number of protein fractions in the blood of different insect larvae: five fractions in *Galleria mellonella*, three fractions in *Macrothylacia rubi* and several fractions in *Bombyx mori*. In the adult of *Dytiscus marginalis* the same author recorded only one fraction. As mentioned in the previous section, the haemolymph of fully grown *Drosophila* larvae contains two protein fractions (WUNDERLY and GLOOR, 1953; CHEN, 1956) and that of *Culex* larva one fraction. According to their mobility and the values of the isoelectric point reported by the above authors, all these protein components seem to be more related to the serum globulins than the serum albumins (KRIEG, 1956). This is certainly an over-simplified way of identifying the proteins in the insect haemolymph. The electrophoretic pattern is not constant from insect to insect and varies to a great extent according to developmental stage even in the same species. The proteins separated on the electropherograms are most likely species specific (DENUCÉ, 1958). Valuable information can be provided by applying the serological technique (FOX and YOON, 1957; WUNDERLY, 1957). More work is also needed to determine the morphogenetic and physiological roles of these protein components.

From previous work it is known that the concentration of free amino-acids and proteins in the insect blood changes considerably during post-embryonic development. Again, the situation varies from insect to insect. As reported previously (CHEN, 1958) and confirmed in this study, the amino-acid concentration of *Culex* larvae does not decrease significantly even at later stages. This is in contrast to *Drosophila* larvae which show a continuous drop in the total amount of free ninhydrin-reacting components with the advance of age (HADORN and STUMM-ZOLLINGER, 1953; CHEN and HADORN, 1955; HADORN, 1956). With regard to protein contents, the values are usually low during earlier development and then rise rapidly towards the end of larval life. In both *C. pipiens* and *C. fatigans* the increase of blood proteins is especially striking near the time of pupation. Similar results have been reported for *Bombyx mori* (DRILHON, 1954; DENUCÉ, 1957), *Galleria mellonella* (DENUCÉ, 1957), and *Drosophila melanogaster* (CHEN, 1956). Hence, for an evaluation of experimental data the developmental age of the material used has to be strictly controlled.

As demonstrated in the present study, mosquito larvae of the anautogenous form (*C. fatigans*) contain about 30 per cent more proteins per unit volume haemolymph than those of the autogenous form (*C. pipiens*). On the other hand, the total blood concentration of the free ninhydrin-positive substances is higher in the autogenous form than in the anautogenous form. There exists apparently a kind of compensation in the contents of blood proteins and free amino-acids. It has to be mentioned that, according to estimations from parallel cultures in this work, *C. pipiens* reached an average fresh weight of 5·0–6·3 mg per larva at the end of larval development and *C. fatigans* only 3·4–4·5 mg per larva reared under equal culture conditions (Fig. 4). The total quantity of protein reserves appears therefore the same in the two strains. Anyway, these data suggest that larvae of the anautogenous form have no serious shortage of protein reserves as compared with those of the autogenous form. The anautogenous mosquitoes definitely have sufficient nutritional reserves to produce a certain number of eggs. This conclusion is in agreement with the observation described by CLEMENTS (1956), who compared the fat body of larvae of the two strains. Therefore, autogeny and anautogeny can hardly be explained by differences in the quantity of reserve materials. A more probable explanation is that in autogenous females, a gonadotropic hormone is released automatically from the corpora allata soon after hatching, whereas in anautogenous females a blood meal is required to initiate the hormone secretion. More recently, evidence has been provided to support this hypothesis by ligation and transplantation experiments (CLEMENTS, 1956; LARSEN, 1958). However, further study is needed to look for histological differences in the endocrine organs between these two forms under comparable conditions, and to prove whether the hypothesis of hormone secretion holds true in all mosquito species.

Acknowledgement—Thanks are due to Prof. E. HADORN for his encouragement and valuable criticism during this work.

REFERENCES

BENZ G. (1957) Untersuchungen über die Wirkung der Letalfaktoren letal-bluter (*lbl*) und letal-polymorph (*lpm*) von *Drosophila melanogaster*. *Z. indukt. Abstamm.- u. VererbLehre* **88**, 78–114.

BODE F. H., HÜBNER H. J., BRÜCKNER H., und HOERES K. (1952) Eine einfache quantitative Bestimmung von Aminosäuren im Papierchromatogramm. *Naturwiss.* **39**, 524–525.

BOISSEZON P. DE (1933) De l'utilisation des protéines et du fer d'origine végétale dans la maturation des œufs chez *Culex pipiens* L. *C.R. Soc. Biol., Paris* **114**, 487–489.

BUCK A. DE (1935) Beitrag zur Rassenfrage bei *Culex pipiens*. *Z. angew. Ent.* **22**, 242–252.

CHEN P. S. (1956) Elektrophoretische Bestimmung des Proteingehaltes im Blut normaler und letaler (*ltr*) Larven von *Drosophila melanogaster*. *Rev. suisse Zool.* **63**, 216–229.

CHEN P. S. (1958) Studies on the protein metabolism of *Culex pipiens* L. I. Metabolic changes of free amino-acids during larval and pupal development. *J. Ins. Physiol.* **2**, 38–51.

CHEN P. S. (1959) Trennung der Blutproteine von *Drosophila*- und *Culex*-Larven mittels Stärke-Elektrophorese. *Rev. suisse Zool.* **66**, 280–289.

CHEN P. S. and HADORN E. (1955) Zur Stoffwechselphysiologie der Mutante letal-meander (*lme*) von *Drosophila melanogaster*. *Rev. suisse Zool.* **62**, 338–347.

CLARK E. W. and BALL G. H. (1956) Preliminary microelectrophoretic studies of insect proteins. *Physiol. Zool.* **29**, 206–212.

CLEMENTS A. N. (1956) Hormonal control of ovary development in mosquitoes. *J. exp. Biol.* **33**, 211–233.

CREMER H. J. and TISELIUS A. (1950) Elektrophorese von Eiweiss in Filtrierpapier. *Biochem. Z.* **320**, 273–283.

DENUCÉ J. M. (1957) Über die Trennung von Hämolymphe-Proteinen mittels einer modifizierten Stärke-Elektrophorese-Methode. *Z. Naturf.* **12b**, 434–436.

DENUCÉ J. M. (1958) Zonenelektrophoretische Untersuchungen der Hämolymphe-Proteine von Insekten in verschiedenen Stadien der Larvenentwicklung. *Z. Naturf.* **13b**, 215–218.

DETINOVA T. S. (1945) On the influence of glands of internal secretion upon the ripening of the gonads and the imaginal diapause of *Anopheles maculipennis*. *Zool. Zh.* **34**, 291–298. In Russian.

DRILHON A. (1954) Etude électrophorétique des protéines de l'hémolymphe du *Bombyx mori* au cours de son cycle de croissance. *C.R. Acad. Sci., Paris* **238**, 2452–2454.

DURRUM E. L. (1950) A microelectrophoretic and microionophoretic technique. *J. Amer. chem. Soc.* **72**, 2943–2948.

FARID M. A. (1949) Relationships between certain populations of *Culex pipiens* Linnaeus and *Culex quinquefasciatus* Say in the United States. *Amer. J. Hyg.* **49**, 83–100.

FISCHER F. G. and DÖRFEL H. (1953) Zur quantitativen Auswertung der Papierchromatogramme von Eiweiss-Hydrolysaten. *Biochem. Z.* **324**, 544–566.

FOX A. S. and YOON S. B. (1957) Application of Agar-diffusion techniques to the analysis of *Drosophila* antigens. *Anat. Rec.* **128**, 552.

GASCHEN H. (1932) Influence de la température et de la nutrition larvaire sur le développement de *Culex pipiens* (race autogène). *Bull. Soc. Pat. exot.* **25**, 577–581.

GILLETTE J. D. (1957) Variation in the time of release of the ovarian development hormone in *Aedes aegypti*. *Nature, Lond.* **180**, 656–657.

GREENBERG J. (1951) Some nutritional requirements of adult mosquitoes (*Aedes aegypti*) for oviposition. *J. Nutr.* **43**, 27–35.

HADORN E. (1956) Patterns of biochemical and developmental pleiotropy. *Cold Spr. Harb. Symp. quant. Biol.* **21**, 363–373.

HADORN E. and STUMM-ZOLLINGER E. (1953) Untersuchungen zur biochemischen Auswirkung der Mutante "*letal-translucida*" (*ltr*) von *Drosophila melanogaster*. *Rev. suisse Zool.* **60**, 506–516.

VOL. 3 1959

HECHT O. (1933) Die Blutnahrung, die Erzeugung der Eier und die Überwinterung der Stechmückenweibchen. *Arch. Schiffs- u. Tropenhyg.* **37**, 125–211.

HOSOI T. (1954) Egg production in *Culex pipiens pallens* Coquillett. III. Growth and degeneration of ovarian follicles. *Jap. J. med. Sci. Biol.* **7**, 111–127.

KRIEG A. (1956) Elektrophoretische Untersuchungen an Hämolympheproteinen von Insekten und anderen Avertebraten. *Naturwiss.* **43**, 60–61.

LARSEN J. R. (1958) Hormone-induced ovarian development in mosquitoes. *Science* **127**, 587–588.

LAVEN H. (1953) Reziprok unterschiedliche Kreuzbarkeit von Stechmücken (*Culicidae*) und ihre Deutung als plasmatische Vererbung. *Z. indukt. Abstamm.- u. VererbLehre* **85**, 118–136.

LAVEN H. (1957) Vererbung durch Kerngene und das Problem der ausserkaryotischen Vererbung bei *Culex pipiens* I. Kernvererbung. *Z. indukt. Abstamm.- u. VererbLehre* **88**, 443–477.

MEDNIKOVA M. V. (1952) The endocrine organs corpora allata and corpora cardiaca of mosquitoes (Fam. *Culicidae*). *Zool. Zh.* **31**, 676–685. In Russian.

MÖLLRING F. K. (1956) Autogene und anautogene Eibildung bei *Culex* L. Zugleich ein Beitrag zur Frage der Unterscheidung autogener und anautogener Weibchen an Hand von Eiröhrenzahl und Flügellänge. *Z. Tropenmed. u. Parasit.* **7**, 15–48.

ROUBAND E. (1933) Essai synthétique sur la vie du moustique commun (*Culex pipiens*). L'évolution humaine et les adaptations biologiques du moustique. *Ann. Sci. nat. (Zool.)* **16**, 5–168.

ROUBAND E. and TOUMANOFF C. (1930) Sur une race physiologique suractive du moustique commun, *Culex pipiens* L. *Bull. Soc. Pat. exot.* **23**, 196–201.

ROZEBOOM L. E. (1951) The *Culex pipiens* complex in North America. *Trans. R. ent. Soc. Lond.* **102**, 343–353.

TWOHY D. W. and ROZEBOOM L. R. (1957) A comparison of food reserves in autogenous and anautogenous *Culex pipiens* populations. *Amer. J. Hyg.* **65**, 316–324.

WUNDERLY CH. (1953) Die Papierelektrophorese. Methoden und Ergebnisse. *Chimia* **7**, 145–160.

WUNDERLY CH. (1957) Die Immunoelektrophorese. *Experientia* **13**, 421–434.

WUNDERLY CH. and GLOOR H. (1953) Versuche zur Charakterisierung der larvalen Blutproteine normaler und letaler Genotypen von *Drosophila* mittels Papier-Elektrophorese. *Protoplasma* **42**, 273–282.

YOELI M. and MER G. G. (1938) The relation of blood feeds to the maturation of ova in *Anopheles elutus*. *Trans. R. Soc. trop. Med. Hyg.* **31**, 437–444.

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 345 *to* 348. *Pergamon Press Ltd., London. Printed in Great Britain*

DERMAL GLANDS AND MUCIN IN THE MOULTING CYCLE OF *RHODNIUS PROLIXUS* STÅL.

W. F. BALDWIN and T. N. SALTHOUSE

Atomic Energy of Canada Limited, Chalk River, Ontario, Canada

(*Received* 16 *December* 1958)

Abstract—The role of the dermal glands in the moulting cycle of *Rhodnius* has been reinvestigated by means of the alcian blue staining technique for mucin. Mucin is secreted by the majority of the dermal glands from the eleventh day after feeding until the time of moulting. The gland sacs become distended with mucin immediately prior to moulting, and this mucin is discharged and forms an inner lubricating layer on the exuvia during ecdysis.

VOL.
3
1959

INTRODUCTION

THE precise function of the dermal glands, and the nature of the secretions produced by them during the moulting of insects, has not been satisfactorily resolved. For instance, WIGGLESWORTH (1933) originally adopted the general view that the dermal glands secrete the moulting fluid; later (1948) he revised these views and concluded that the moulting fluid originates in the general epidermal layer, and that the dermal glands secrete a cement layer. These arguments are based on the fact that the dermal glands reach the height of their activity at the end of the moulting process, and in WIGGLESWORTH's opinion, apparently discharge their contents after ecdysis (1947). He describes the moulting fluid as a neutral salt-free fluid with proteins in solution, containing a protease and probably a chitinase; its chief function is claimed to be the digestion of the inner layers of the old cuticle. More recent work by PASSONEAU and WILLIAMS (1953) confirms the presence of enzymes capable of digesting the inner layers of the cuticle, and they show the presence of a gel in the moulting fluid during the early days of moulting. These authors, and others, doubted whether moulting fluid would function also as a lubricant during ecdysis, although earlier workers, such as HOOP (1933), were convinced that it was involved in this role. The recent development of selective stains for acid mucopolysaccharides and mucin has enabled the authors to reinvestigate this problem, and to assign to the majority of the dermal glands a definite function in the moulting of *Rhodnius*.

METHODS

Several workers have been interested in the problem of detecting acid mucopolysaccharides and mucin in the tissues of animals during the last decade. In 1950, STEEDMAN introduced the alcian blue method for the staining and detection of mucin in sections of tissue. It was concluded by VIALLI (1951) that alcian blue was a reliable method for staining epithelial mucous and mucoid cells and for the

study of mucopolysaccharides. Later, Lison (1954) stated that "it appears that all structures readily stainable with alcian blue are related to mucopolysaccharides, though the nature of the selectivity of alcian blue is unknown". Wagner and Shapiro (1957) have shown that, when used under controlled conditions, alcian blue can be employed as a reliable indicator for the presence of acid mucopolysaccharides, including mucin.

This study of the dermal glands in the body wall of an insect was done with fourth instar nymphs of the blood-sucking bug *Rhodnius prolixus*. The insects were killed and sectioned at daily intervals after a meal of rabbit blood. The posterior half of the abdomen of each insect was fixed in Carnoy's solution, and in some cases, formol alcohol and Eltringham's fixative. The tissues were embedded in paraffin wax on the completion of fixation and dehydration. Sections of 4 and 6 μ thickness were mounted on slides and stained by the following method:

Sections were treated by immersing the slides in a solution of 0·1 per cent alcian blue* in 1 per cent acetic acid for a maximum of 5 min. After several rinses in distilled water, they were moved to alkaline alcohol (95 per cent ethanol with 0·5 per cent ammonia solution), thus converting any alcian blue taken up by tissues into an insoluble material and preventing its loss during subsequent counter-staining. After thorough rinsing with distilled water, the nuclei were stained for 10 to 15 min with nuclear fast red (0·1 g of nuclear fast red in 100 ml of 5 per cent aluminium sulphate). Cytoplasmic tissues were counter-stained with 0·1 per cent metanil yellow. This staining procedure imparts a brilliant blue-green coloration to mucins, with red nuclei and yellow cytoplasmic elements.

RESULTS

The development of the cuticle and the whole moulting process in *Rhodnius* has been thoroughly investigated by Wigglesworth (1933, 1948). The first evidence of the development of a new epicuticle in fourth instar larvae is found under the old cuticle at the eighth or ninth day after feeding. By the eleventh day, developing dermal glands can be recognized in the epidermal layer below the newly formed epicuticle. Many of these glands contain small amounts of a clear hyaline secretion which gives a positive staining reaction for mucin when treated by the methods given above (Figs. 1 and 2). The evidence of the presence of mucin in these glands given by alcian blue was further confirmed by positive staining with Mayer's mucicarmine and metachromatic staining with toluidine blue (Lillie, 1954); these tests were positive when formol alcohol and Eltringham's solution were substituted as fixing agents. In addition, the contents of the dermal glands lost their staining properties with alcian blue when sections were digested with bovine testicular hyaluronidase† solutions (at 1 mg/ml in 0·8 per cent saline and pH 7·2) and with bovine liver β-glucuronidase† (at 1 mg/ml in 0·8 per cent saline at pH 4) (Pearse, 1953), suggesting that the substance is a hyaluronic

* Alcian blue supplied by G. T. Gurr, Ltd., London.

† Enzymes supplied by Worthington Biochemical Corp., Freehold, N.J.

VOL.
3
1959

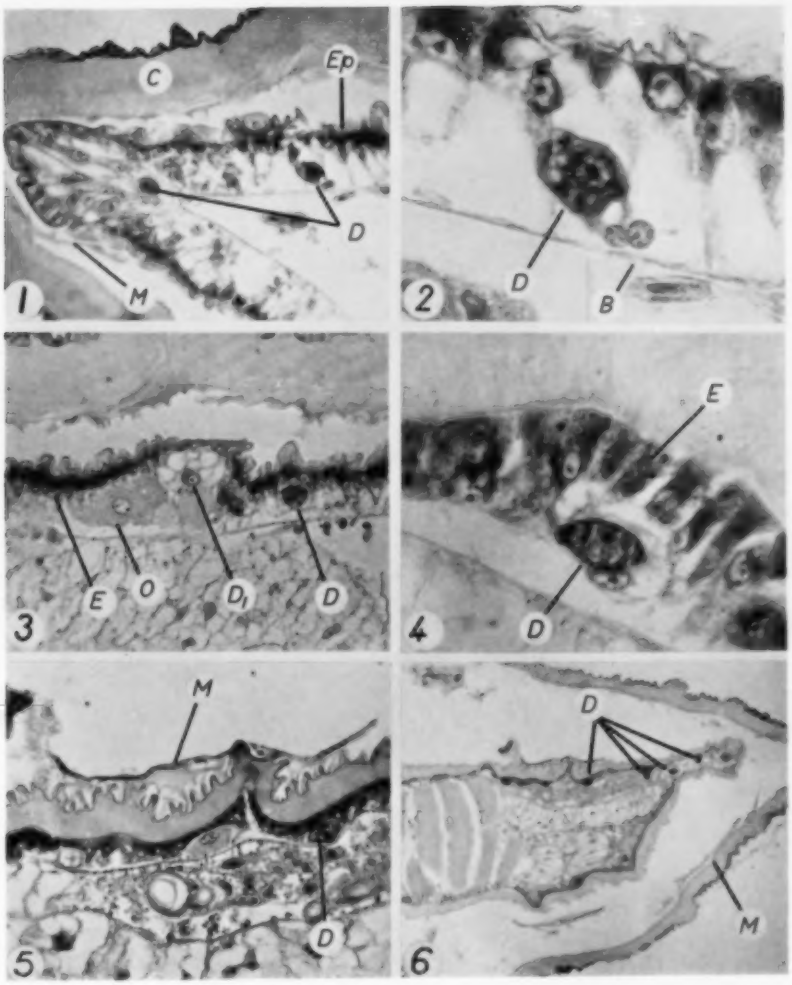


Plate I

FIG. 1. Section through lateral edge of abdomen at 11 days after blood meal showing newly formed epicuticle (*EP*), dermal glands with mucin (*D*) and traces of mucin in ecdysial space (*M*). Old outer cuticle (*C*). (× 300.)

FIG. 2. Enlarged view of individual dermal gland (*D*) from Fig. 1 showing duct leading to surface of epicuticle. Gland is actively secreting mucin. (×1200.)

FIG. 3. Section through abdominal wall showing old cuticle above, epidermal cell layer (*E*), oenocyte (*O*), nonmucin producing dermal gland (D_1), and another dermal gland (*D*) containing mucin. (× 300.)

FIG. 4. Dermal gland at 13 days after feeding showing sac distended with contained mucin; nucleus of dermal gland cell displaced. Epidermal layer (*E*) negative for mucin. (× 800.)

FIG. 5. Section at 14 days showing mucin (*M*) on surface of new epicuticle. Dermal gland (*D*). (× 250.)

FIG. 6. Dermal glands (*D*) distended with mucin at 14 days, a few hours before moulting. Traces of mucin (*M*) on inner surface of the old cuticle. (× 100.)

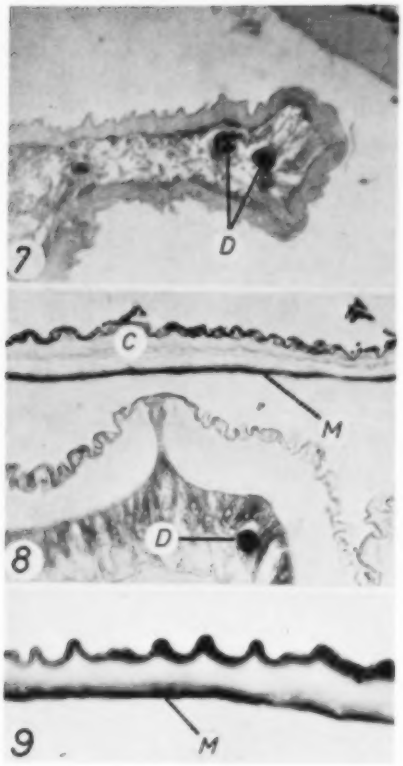


Plate II

FIG. 7. Enlarged view of dermal glands a few hours before moulting. (× 200.)

FIG. 8. During ecdysis; dermal gland partially emptied (*D*); mucin deposit (*C*) lining exuvia almost complete. (× 300.)

FIG. 9. Section through exuvia immediately after moulting showing mucin layer (*M*). (× 400.)

Note: All sections were stained with alcian blue and counterstained with nuclear fast red and metanil yellow. Only dermal glands (*D*) and mucin (*M*) gave a positive reaction with alcian blue; nuclei were stained deep red and other elements (i.e. cytoplasm of epidermal cells, etc.) varying shades of pale yellow.

acid-containing mucin. The dermal glands, although somewhat more numerous in the lateral abdominal walls (Fig. 6), are otherwise evenly distributed throughout the whole of the body epidermis. The glands are spaced approximately 0·15–0·30 mm apart, with about 25 mucin producing dermal glands per square millimetre. It was significant, however, that a small number of glands giving a negative result with alcian blue were present throughout the epidermis (Fig. 3, *D*). From the eleventh day onward, increasing amounts of a substance giving the same staining reactions as the majority of the dermal glands was found in the exuvial space between the old and new cuticles (Figs. 1, 5 and 6).

On succeeding days, distended ovoid sacs containing mucin are formed within the dermal glands themselves (Figs. 4, 5 and 7). At 13 days, or, within a day of moulting, these sacs reach an average length of 15–20 μ. Simultaneously, the old cuticle becomes much thinner because of the digestion of the endocuticle by enzymatic action (PASSONEAU and WILLIAMS, 1953) (Fig. 6); also, a thin deposit giving a positive reaction to alcian blue begins to form on the inner surface of the remains of the old cuticle (Fig. 8). The new epicuticle develops a thin, outer, waxy layer, the "wax layer" discussed by WIGGLESWORTH (1933). The presence of this layer was shown in our studies by various fat soluble stains on carbowax embedded sections; it was found that the wax layer was highly repellent to aqueous solutions. Immediately before ecdysis, the dermal glands are fully distended with mucin; after the old cuticle is discarded, only slight traces of mucin remain in the glands. When exuviae were fixed, sectioned, and stained with alcian blue, the mucin could be seen as a thin layer 2 μ in thickness (Fig. 9). This layer gave the same staining and enzyme digestion reactions as the mucin in the dermal glands. We believe, from this histological evidence, that the final discharge of mucin from the dermal glands condenses on the inner surface of the old cuticle and forms a lubricating film against the outer waxy layer of the new epicuticle during ecdysis.

VOL. 3 1959

CONCLUSIONS

This work has shown that the majority of dermal glands in the epidermis of *Rhodnius* produce a positive mucin staining material, and this may be the sole function of these glands which stain with alcian blue. Enzyme digestion tests indicating the presence of hyaluronic acid support the view that these dermal glands secrete a mucin. It seems unlikely that both mucin and enzymes are secreted by the same cells, and it is possible that the function of the small number of glands with no evidence of mucin before moulting is to secrete cuticle digesting enzymes. The dermal glands were found to be empty but distended after ecdysis; this suggests that some internal pressure mechanism may empty the sacs. Mucin can be detected as a condensed layer on the inner surface of the old cuticle, where it undoubtedly acts as a lubricating layer against the wax layer of the new epicuticle during moulting.

Acknowledgement—The authors wish to express their appreciation to Prof. V. B. WIGGLESWORTH for making available the original stock of *Rhodnius* used in this work, and for his comments on our original manuscript.

REFERENCES

HOOP M. (1933) Hautungshistologie einiger Insekten. *Zool. Jb.* (*Anat.*) **57**, 433–464.

LILLIE R. D. (1954) *Histopathologic Technic and Practical Histochemistry*. Blakiston Co. New York.

LISON L. (1954) Alcian blue with chlorantine fast red B. A technique for selective staining of mucopolysaccharides. *Stain Tech.* **29**, 131–138.

PASSONEAU J. V. and WILLIAMS C. M. (1953) The moulting fluid of the Cecropia silkworm. *J. exp. Biol.* **30**, 545–560.

PEARSE A. G. E. (1953) *Histochemistry*. Churchill, London.

STEEDMAN H. F. (1950) Alcian blue, 8GS.: A new stain for mucin. *Quart. J. micr. Sci.* **91**, 477–479.

VIALLI M. (1951) Observazioni sull' uso dell' alcian blue 8GS. nello studio dei muco-polisaccaridi. *Boll. Soc. ital. Biol. sper.* **27**, 297–298.

WAGNER B. M. and SHAPIRO S. H. (1957) Application of alcian blue as a histochemical method. *Lab. Invest.* **6**, 472–477.

WIGGLESWORTH V. B. (1933) The physiology of the cuticle and of ecdysis in *Rhodnius prolixus* (Triatomidae, Hemiptera). *Quart. J. micr. Sci.* **16**, 269–318.

WIGGLESWORTH V. B. (1947) The epicuticle in an insect, *Rhodnius prolixus* (Hemiptera). *Proc. roy. Soc.* (B) **134**, 163–181.

WIGGLESWORTH V. B. (1948) The insect cuticle. *Biol. Rev.* **23**, 409–451.

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 349 *to* 356. *Pergamon Press Ltd., London. Printed in Great Britain*

METABOLISM OF OXIMES IN THE SILKWORM *BOMBYX MORI* L.—I INHIBITION OF TRANSAMINASES*

B. BHEEMESWAR† and P. FAULKNER

Laboratory of Insect Pathology, Sault Ste. Marie, Ontario, Canada

(*Received* 19 *January* 1959)

Abstract—Several oximes were found to inhibit the glutamic-aspartic and glutamic-alanine transaminases of the silkworm, *Bombyx mori* L. as well as the glutamic-aspartic transaminases of rabbit liver and *Escherichia coli*. Kinetic studies indicate that the inhibition by pyruvic oxime of silkworm glutamic-aspartic transaminases is not reversed by either α-ketoglutarate or pyridoxal phosphate, but some reversal occurs with aspartate. Aspartic decarboxylase of the silkworm is also inhibited by pyruvic oxime. We were not able to demonstrate transoximase or an enzyme hydrolysing pyruvic oxime in silkworm extracts.

VOL. 3 1959

INTRODUCTION

OXIMES of keto acids and of sugars are metabolized in a variety of mammalian tissues, in silkworm preparations, and in micro-organisms. The following four types of enzymes which participate in these reactions have been described: (a) Oximase, which reduces oximes to amino compounds, is present in mammalian tissues, in algae, and in gut-free preparations of silkworm larvae (YAMAFUJI, KAWAKAMI and SHINOHARA, 1952b; YAMAFUJI *et al.*, 1954; YAMAFUJI *et al.*, 1956a). (b) Pyruvic oxime oxidase catalyses the decomposition of the oxime into pyruvic acid and nitrate by soil micro-organisms (QUASTEL *et al.*, 1952). (c) *Tetrahymena pyriformis* contains an enzyme, which has been partially purified, that catalyses hydrolysis of pyruvic oxime to pyruvic acid and hydroxylamine (SEAMAN, 1954, 1957). (d) Transoximase has been described by YAMAFUJI *et al.* (1953b) and by YAMAFUJI *et al.* (1956b). This enzyme, which has been found in silkworm larvae and a few mammalian tissues, catalyses the transfer of the oxime group from such substances as glucose oxime, pyruvic oxime, and acetoxime to acceptor carbonyl compounds. For example, pyruvic oxime transfers its oxime group to a mixture of acetaldehyde and acetone or to α-ketoglutarate (YAMAFUJI and AKITA, 1952a). Silkworm tissue extracts also contain a group of transaminases (BHEEMESWAR and SREENIVASAYA 1952; KOIDE *et al.*, 1955; KOIDE *et al.*, 1956; FUKUDA, 1956, 1957), and we were interested in comparing transoximase with these transaminases. In the preliminary work, it soon became apparent that oximes were powerful inhibitors

* Contribution No. 495, Forest Biology Division, Science Service, Department of Agriculture, Ottawa, Canada.

† Post Doctoral Research Fellow, National Research Council, Ottawa, Canada.

of transaminases and in this study we have investigated the nature and specificity of this inhibition in an attempt to assess the biological function of pyruvic oxime in the silkworm.

MATERIALS

Pyruvic oxime was prepared by reacting 1·0 g of sodium pyruvate (Nutritional Biochemical Corporation, Cleveland, Ohio) with 4·0 g hydroxylamine hydrochloride and 4·0 g sodium acetate in about 5 ml water. The mixture was heated in a boiling water bath for 10 min and then cooled in ice. Crystals appeared on scratching the vessel with a glass rod and they were washed repeatedly with cold absolute alcohol to remove hydroxylamine, and dried over silica gel. The resulting needle shaped crystals had a sharp m.p. 183–4°C and the yield was 35 per cent of the theoretical. The preparation was free from hydroxylamine and pyruvate as checked by chromatographic and enzymatic assays.

α-Ketoglutaric oxime was prepared according to Wood *et al.* (1948). The crystals were further purified by washing with 2 N HC1 to remove excess hydroxylamine and the residue dried over silica gel. The preparation was checked for purity by chromatography, and the final product had a m.p. 119–122°C. The yield was 20 per cent of the theoretical.

Mannose, ribose, and acetone oximes were prepared according to Wolfrom and Thompson (1931) and were recrystallized twice from 60 per cent methanol. These products were chromatographically pure and had m.p. 182–185, 133–136, and 40–43°C respectively.

Reduced diphosphopyridine nucleotide (DPNH) was the purified grade of the California Research Foundation, Los Angeles, California.

Malic and lactic dehydrogenases, twice crystallized from rabbit muscle, were obtained from Worthington Biochemical Corporation, Freehold, N.J., and stored at 4°C.

The silkworm transoximase preparation was obtained by homogenizing the gut-free tissues of the silkworm in a Waring blendor with 5 volumes of M/15 phosphate buffer of pH 5·6 and was stored at — 10°C.

The silkworm transaminase preparation was obtained by homogenizing the gut- and gland-free tissues in 5 volumes of M/15 phosphate buffer, pH 7·4. The homogenate was kept at 4°C for 3–4 hr, then centrifuged, and the clear straw-coloured supernatant was stored at — 10°C.

Liver transaminase was obtained from a nembutal-anaesthetized rabbit. The ground liver was extracted in a Potter-Elvehjem homogenizer with M/15 phosphate buffer of pH 7·4. The clear centrifugate was dialysed against water at 4° for 18 hr and stored at −10°C.

Transaminase from E. coli: Washed and dehydrated *E. coli* cells (50 mg) previously grown in nutrient broth were extracted in a Mickle disintegrator with 20 g glass beads and 10 ml of M/15 phosphate buffer, pH 7·4. The opalescent extract was stored at — 10°C.

METHODS

Transoximase was assayed by measuring pyruvate produced as a result of the transference of the oxime group from pyruvic oxime to a mixture of acetone and acetaldehyde at pH 5·6. (YAMAFUJI *et al.*, 1953b). Pyruvate was determined spectrophotometrically at 340 mμ in the presence of lactic dehydrogenase and DPNH.

Glutamic-aspartic and glutamic-alanine transaminase activities were assayed spectrophotometrically using malic and lactic dehydrogenases and DPNH (KARMEN, 1955). The test system for glutamic-aspartic transaminase activity consisted of 33 μM L-aspartate, 33 μM α-ketoglutarate, 0·25 μM DPNH, 330 μM phosphate buffer (pH 7·4), 0·2 ml malic dehydrogenase (1:10), and 0·1 ml transaminase preparation in a total volume of 3·0 ml. The mixture was incubated at room temperature (21°C). The decrease in optical density at 340 mμ was measured at 30 sec intervals. A unit of transaminase was taken as that amount of enzyme which causes a decrease of 0·001 optical density units per minute under the conditions specified above. The glutamic-alanine transaminase test system consisted of 66 μM DL-alanine, 33 μM α-ketoglutarate, 330 μM phosphate buffer (pH 7·4), 0·25 μM DPNH, 0·2 ml lactic dehydrogenase (1:100), and 0·1 ml suitably diluted transaminase preparation.

VOL. 3 1959

Decarboxylase activity was assayed by measuring the volume of carbon dioxide produced as a result of the enzymatic decarboxylation of L-aspartate at pH 5·0 in 0·2 M acetic-acetate buffer at 37°C in a conventional Warburg instrument with air as the gas phase. The assay system consisted of 100 μM aspartate, 1·0 μM α-ketoglutarate as the activator (BHEEMESWAR, 1955) and 40 μM buffer in a total volume of 1·0 ml. The microlitre gas evolved during 60 min incubation following a 10-min equilibration period was taken as a measure of the decarboxylase activity.

Oximes and amino acids were separated by ascending chromatography using Whatman No. 1 filter paper. The chromatographic solvent for separation of the keto acid oximes (YAMAFUJI and ETO, 1953a) contained amyl acetate, 80 per cent formic acid, and water (4:1:1 by volume, upper layer). The spots were detected by spraying the paper with 1·0 per cent alcoholic solution of $FeCl_3$. Ribose and mannose oximes were separated in ethyl acetate-acetic acid 50/50 v/v., and the spots were detected with ammoniacal silver nitrate. Amino acids were separated chromatographically in methanol-water-pyridine 80:20:4 by volume (REDFIELD, 1953) and detected by the ninhydrin reaction.

RESULTS

It was found that the spectrophotometric method of assaying transaminase was suitable in these studies. The rate of oxidation of DPNH by malic and lactic dehydrogenases in the presence of oxalacetate and pyruvate was unaffected by several oximes, indicating that these dehydrogenases are not inhibited by oximes (Fig. 1).

Transoximase activity was, however, not detected in our silkworm preparations when they were incubated in the standard test system from 4 to 16 hr at 35°C. Under our assay conditions we could have measured the formation of as little as 0·01 μM pyruvate.

The addition of malic dehydrogenase was found to be unnecessary in the assay of glutamic-aspartic transaminase activity as a DPN-specific malic dehydrogenase was present in all our transaminase preparations in optimal concentrations. It was established that the rate of decrease of optical density was proportional to the length of incubation and the enzyme concentration with the various transaminase preparations used.

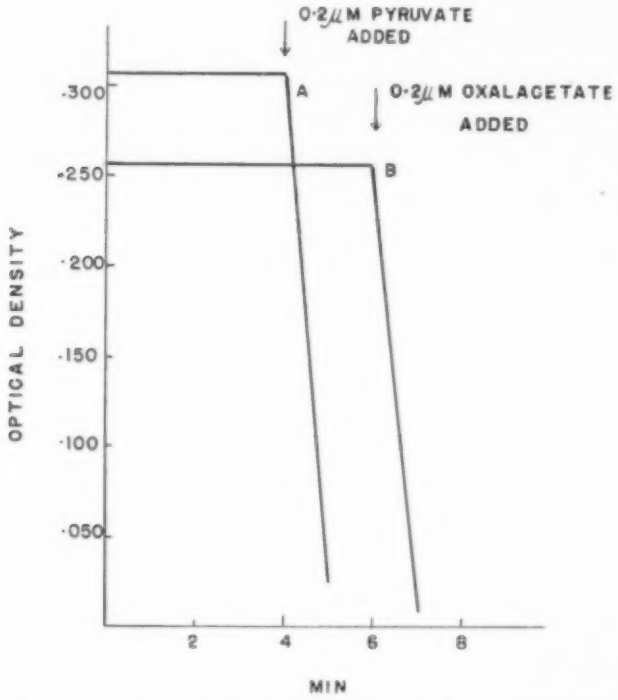


FIG. 1. Test system *A* contained 330 μM phosphate buffer of pH 7·4, 0·25 μM DPNH, 0·2 ml lactic dehydrogenase, and 1·0 μM pyruvic oxime in a total volume of 2·9 ml. At the end of 4 min 0·2 μM pyruvate was added. System *B* contained 330 μM phosphate buffer of pH 7·4, 0·25 μM DPNH, 0·2 ml malic dehydrogenase, and 1·0 μM pyruvic oxime in a total volume of 2·9 ml. At the end of 6 min 0·2 μM oxalacetate was added.

The data in Table 1 show that oximes such as those of pyruvic acid, α-keto-glutaric acid, ribose, mannose, and acetone are inhibitors of silkworm glutamic-aspartic transaminase. Of those mentioned pyruvic oxime is the most effective. Pyruvic oxime also inhibits two other amino acid-utilizing enzymes in the silkworm, namely glutamic-alanine transaminase, and aspartic decarboxylase. A comparison is given in Table 2 of the effect of pyruvic oxime on the glutamic-aspartic, glutamic-alanine transaminases and aspartic decarboxylase of the silkworm. The transaminases are considerably more sensitive to the oxime than is aspartic decarboxylase.

TABLE 1. THE INFLUENCE OF OXIMES ON SILKWORM GLUTAMIC-ASPARTIC TRANSAMINASE

Oximes	Concentration of inhibitor (M)	Percentage inhibition
Pyruvic oxime	$3{\cdot}33 \times 10^{-4}$	79·0
α–Ketoglutaric oxime	$3{\cdot}33 \times 10^{-4}$	6·8
α–Ketoglutaric oxime	$1{\cdot}66 \times 10^{-3}$	50·1
Ribose oxime	$3{\cdot}33 \times 10^{-4}$	57·1
Acetone oxime	$3{\cdot}33 \times 10^{-3}$	60·0
Mannose oxime	$1{\cdot}66 \times 10^{-4}$	12·1

TABLE 2. THE INHIBITION OF THREE SILKWORM ENZYMES BY PYRUVIC OXIME

Enzymes	Concentration of inhibitor	Percentage inhibition
Glutamic-aspartic transaminase	$1{\cdot}0 \times 10^{-5}$	29·8
	$1{\cdot}0 \times 10^{-4}$	85·5
Glutamic-alanine transaminase	$3{\cdot}33 \times 10^{-6}$	66·6
	$6{\cdot}66 \times 10^{-6}$	100·0
Aspartic decarboxylase	$7{\cdot}5 \times 10^{-3}$	79·0
	$1{\cdot}0 \times 10^{-2}$	100·0

It was of interest to determine whether preparations having transaminase activity and obtained from sources other than insects were also inhibited by oximes. In Table 3 the effects of pyruvic oxime, α-ketoglutaric oxime, and hydroxylamine, all at the same molar concentration, have been compared with preparations having glutamic-aspartic activity obtained from *E. coli*, rabbit liver, and the silkworm. The inhibitions due to pyruvic oxime and hydroxylamine on the transaminases from all three sources are of a comparable order. Rabbit liver transaminase is, however, more sensitive to α-ketoglutaric oxime than are transaminases obtained from *E. coli* and the silkworm.

TABLE 3. INFLUENCE OF PYRUVIC OXIME, α–KETOGLUTARIC OXIME, AND HYDROXYLAMINE ON GLUTAMIC-ASPARTIC TRANSAMINASES FROM VARIOUS SOURCES

Source of enzyme	Percentage inhibition at $33{\cdot}3 \times 10^{-4}$ M by pyruvic oxime	α–ketoglutaric oxime	hydroxylamine
E. coli	86·0	11·5	84·0
Rabbit liver	73·0	60·8	85·0
Silkworm	73·0	6·8	84·0

Kinetic studies on the silkworm glutamic-aspartic transaminase show that when the reciprocal of the rate of the transamination reaction is plotted against the reciprocal of the substrate concentration according to the method of

LINEWEAVER and BURK (1934) a straight line is obtained (Fig. 2). The intercept with the abscissa is the reciprocal of the Michaelis constant (K_s). Values of $6{\cdot}66 \times 10^{-4}$ M for aspartate (Fig 2a) and $5{\cdot}0 \times 10^{-5}$ M for α-ketoglutarate (Fig. 2b)

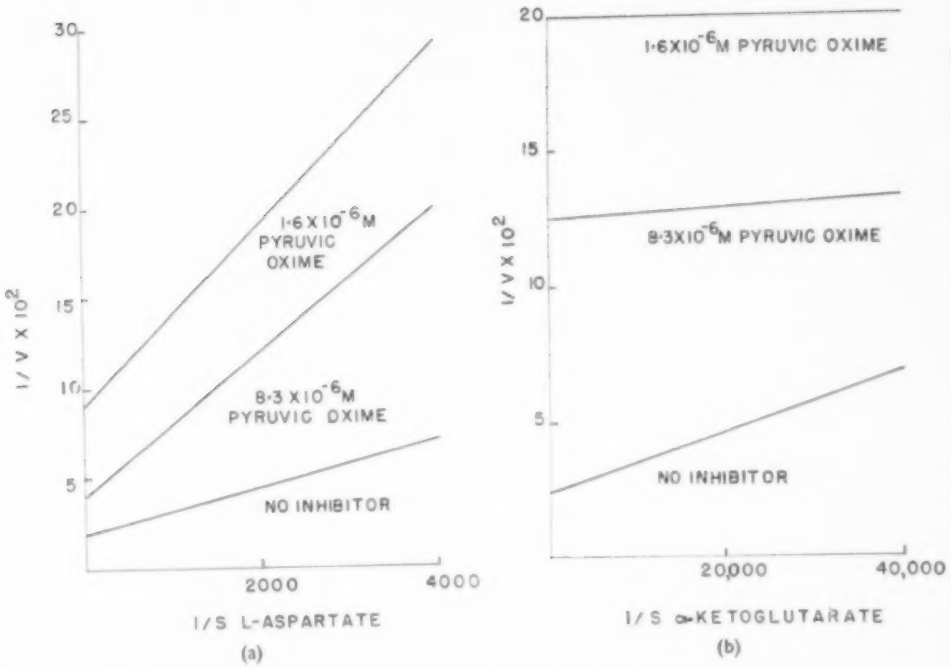


FIG. 2. (a) Standard test system for glutamic-aspartic transaminase as indicated in the text with concentrations of L-aspartate and pyruvic oxime varied as indicated.

(b) Standard test system for glutamic-aspartic transaminase as indicated in the text with concentrations of α-ketoglutarate and pyruvic oxime varied as indicated.

were obtained. Reaction rates plotted in the presence of various concentrations of pyruvic oxime indicate that inhibition is non-competitive with respect to either of the substrates although some reversal is obtained with aspartate. Pyridoxal phosphate is ineffective in reversing the inhibition, which suggests that pyruvic oxime does not form a complex with the coenzyme.

DISCUSSION

We have been unable to demonstrate pyruvate formation from pyruvic oxime in silkworm tissue extracts, and we conclude that neither transoximation nor pyruvic oxime hydrolysis take place in them. It is possible however that our method of extraction could have inactivated enzymes responsible for pyruvic oxime metabolism. Accordingly we report on the *in vivo* metabolism of pyruvic oxime in the following paper.

Our main *in vitro* results with pyruvic and other oximes have been to demonstrate that they are inhibitors of three enzymes associated with amino acid metabolism, i.e. glutamic-aspartic and glutamic-alanine transaminases and aspartic decarboxylase, but have no effect on dehydrogenases such as those of malic and lactic acid. In particular, pyruvic oxime is as powerful an inhibitor of transaminase as is hydroxylamine. As pointed out by GUNSALUS and STAMER (1955), kinetic studies on transaminases in which there are four reactants as well as two coenzymes are extremely difficult to interpret and we are unable to account for the mechanism of oxime inhibition of transaminases.

REFERENCES

BHEEMESWAR B. (1955) Studies on transaminase and decarboxylase catalysed by extracts of the silkworm, *Bombyx mori* L. *Nature, Lond.* **176**, 555–556.

BHEEMESWAR B. and SREENIVASAYA M. (1952) Occurrence of transaminase in silkworm *Bombyx mori* L. *Curr. Sci.* **21**, 253–255.

FUKUDA T. (1956) Conversion of pyruvic acid to alanine in the silkworm larvae. *Nature, Lond.* **180**, 245.

FUKUDA T. (1957) Biochemical studies on the formation of silk protein. IV. Conversion of pyruvic acid to alanine in the silkworm larvae. *J. Biochem., Tokyo* **44**, 505–510.

GUNSALUS I. C. and STAMER J. R. (1955) Transaminases in Bacteria. In *Methods in Enzymology* **2**, 170. Academic Press, New York.

KARMEN A. (1955) A note on the spectrophotometric assay of glutamic-oxalacetic transaminase in human blood serum. *J. clin. Invest.* **34**, 131–134.

KOIDE F., NAGAYAMA H., and SHIMURA K. (1955) Transaminase from silkworm tissues. *Nippon Nogei-Kagaku Kaishi* **29**, 987–990.

KOIDE F., SHISHIDO T., NAGAYAMA H., and SHIMURA K. (1956) Formation of glycine in the silkworm by transamination. *J. agric. chem. Soc. Japan* **30**, 283–287.

LINEWEAVER H. and BURK D. (1934) Determination of enzyme dissociation constants. *J. Amer. chem. Soc.* **56**, 658–666.

QUASTEL J. H., SCHOLEFIELD P. G., and STEVENSON J. W. (1952) Oxidation of pyruvic oxime by soil micro-organisms. *Biochem. J.* **51**, 278–284.

REDFIELD R. R. (1953) Two-dimensional paper chromatographic systems with high resolving power for amino acids. *Biochim. Biophys. Acta* **10**, 344–345.

SEAMAN G. R. (1954) Pyruvate oxidation by extracts of *Tetrahymena pyriformis*. *J. gen. Microbiol.* **11**, 300–306.

SEAMAN G. R. (1957) The metabolism of pyruvic oxime by extracts of *Tetrahymena pyriformis* S. *Biochim. Biophys. Acta* **26**, 313–317.

WOLFROM M. L. and THOMPSON A. (1931) The reactive form of glucose oxime. *J. Amer. chem. Soc.* **53**, 622–632.

WOOD J. G., HONE M. R., MATTNER M. E., and SYMONS C. P. (1948) Studies on the nitrogen metabolism of plants, VII. Toxicity of some oximes and oximino acids to azotobacter and their utilization. *Austr. J. sci. Res.* (B) **1**, 38–49.

YAMAFUJI K. and AKITA T. (1952a) On transoximation. *Enzymologia* **15**, 313–318.

YAMAFUJI K., KAWAKAMI T., and SHINOHARA K. (1952b) On an enzyme in silkworm which catalyses the transformation of oximes into amino compounds. *Enzymologia* **15**, 199–203.

VOL. 3 1959

YAMAFUJI K. and ETO M. (1953a) Chromatographic study of transoximase. *Enzymologia* **16**, 247–255.

YAMAFUJI K., OMURA H., and MIURA M. (1953b) On the transoximase. *Enzymologia* **16**, 75–80.

YAMAFUJI K., SHIMAMURA M., and TAKAHASHI H. (1954) Oximase and transoximase in green algae. *Enzymologia* **17**, 110–112.

YAMAFUJI K., AOKI M., and OMURA H. (1956a) Improved method of estimating oximase. *Enzymologia* **17**, 371–377.

YAMAFUJI K., SHIMAMURA M., and OMURA H. (1956b) Measurement of transoximase action. *Enzymologia* **17**, 359–362.

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 357 *to* 366. *Pergamon Press Ltd., London. Printed in Great Britain*

THE 24-HR RHYTHM OF EMERGENCE OF *EPHESTIA KÜHNIELLA* ZELL. FROM THE PUPA

F. MORIARTY

Dept. of Zoology, Imperial College, London*

(*Received* 26 *January* 1959)

Abstract—The endogenous 24-hr emergence rhythm of the moth *Ephestia kühniella* Zell. was unaffected by a daily variation of atmospheric pressure or humidity. Decrease in temperature from 30° to 25°C initiated a new rhythm of emergence; the opposite change did not affect the existing emergence rhythm. A change from light to darkness stimulated emergence; a change from darkness to light retarded it. A single decrease of temperature or change of light intensity initiated a new rhythm of emergence. A 16-hr periodicity of emergence was initiated by a corresponding periodicity of temperature but the rhythm reverted to a 24-hr periodicity immediately the temperature variations ceased. No 24-hr rhythm of oxygen consumption by the pupa in uniform conditions could be detected. The time between peak periods of emergence appeared to increase when pupae were kept in constant conditions in a gas mixture containing 7 per cent oxygen. The endogenous emergence rhythm was displaced by about 12 hr after pupae had been kept at 0·5°C for 12 hr.

VOL.
3
1959

INTRODUCTION

BREMER (1926) found that the moth *Ephestia kühniella* Zell. exhibits a 24-hr rhythm of emergence from the pupa, with most emergences in the evening. SCOTT (1936) showed that this rhythm is endogenous (as defined by PARK, 1941) and is influenced by variations of temperature. He also found that the variations of light intensity apparently affect the time of emergence, although he was unable to dissociate light variation completely from temperature variation. This paper is a further study of factors influencing emergence.

MATERIAL AND METHODS

A culture of *E. kühniella* was obtained from those maintained at the Imperial College Field Station, Silwood Park, Berks., and the moths were reared in muslin-covered glass jars containing a mixture of whole-meal flour, middlings and glycerol. They were kept in a constant temperature room at 25 ± 0·1°C, and at a relative humidity of 65 ± 5 per cent. Lighting was variable; usually two 60 W bulbs were on, but for some experiments the room was in darkness. To facilitate the collection of pupae, strips of corrugated cardboard were rolled up and placed in the culture jars, so that many of the larvae pupated in the interstices of the cardboard.

For experiments the pupae were kept in 5-in. petri dishes, placed in circular glass troughs covered with glass plates and containing 15 per cent aqueous potassium hydroxide, which absorbed carbon dioxide and maintained a relative

* Present address: School of Agriculture, Cambridge.

humidity of about 85 per cent (although preliminary experiments showed no evidence for changes of humidity affecting emergence).

The number of newly emerged adults was counted every 4 hr. Where it is stated that the pupae were kept in constant darkness, actually they were exposed to light for a short while each time the number of new adults was counted. All times are stated in terms of the 24-hr clock, and are G.M.T.

Effect of temperature

Two groups of pupae were kept for ten days in normal laboratory conditions (14–24°C). Both groups were then placed in continuous darkness at a constant temperature, one at 25°C, the other at 30°C. Both groups showed a normal endogenous emergence rhythm, the maximum rate of emergence occurring at about 17.00 hr each day.

At 5.00 hr on the fourth day of observation, the pupae were transferred from the one constant temperature to the other. The rhythm was unaffected by an increase of temperature, but in the pupae that were removed to a lower temperature there was a change in the time of day at which most adults emerged, the greatest number recurring at about the time when the pupae had been transferred to the lower temperature (Table 1). It seemed probable that this change depended on

Table 1. Effect of change of temperature on the number of adults emerging

Experimental conditions	Time of observations					
	9	13	17	21	1	5
Increase of temperature:						
pupae at 25°C for 3 days	8	39	71	46	38	21
pupae transferred to 30°C at 5.00 hr for 3 days	43	67	166	148	62	36
Decrease of temperature:						
pupae at 30°C for 3 days	21	35	26	155	59	45
pupae transferred to 25°C at 5.00 hr for 3 days	124	63	52	32	32	124

the time of day at which the temperature was lowered. However, it was possible that this alteration in time of the maximum rate of emergence was due to a temporary upset of the metabolism before acclimatization to the new temperature, and that, whatever the time of day at which transference occurred, there would still be a similar displacement of the peak period by about 10 hr. To test this possibility, two groups of pupae were kept in continuous darkness at 30°C. After three days, when it was clear that the maximum rate of emergence occurred during the period 13–17 hr, one group was transferred to 25°C at 1.00 hr, the other at 9.00 hr. In both groups the subsequent time of maximum emergence occurred at about that time of day when they were transferred to the lower temperature.

These results indicate that the fall of temperature is a stimulus for emergence, and that a single such fall is sufficient to start a new endogenous rhythm of emergence. These conclusions suggested that the 16-hr rhythm of emergence associated with a corresponding periodicity of temperature variations (SCOTT, 1936) would not continue in a constant temperature, but would revert to a 24-hr endogenous rhythm. This was confirmed by the following experiment. Two groups of pupae were kept for alternate 8-hr periods at 25°C and 30°C, but always in darkness. After five days the pupae were kept at a constant temperature. Observations were started on the third day. While the temperature had a 16-hr periodicity the emergence showed a similar periodicity (Table 2a). The emergence rhythm reverted to a 24-hr periodicity when the temperature was kept constant (Table 2b): the peak period of emergence occurred about every 24 hr after the last fall in temperature, which was at 9.00 hr for lot *A*, and at 1.00 hr for lot *B*.

TABLE 2*a*. EMERGENCE WITH 16-HR PERIODICITY OF TEMPERATURE ALTERNATION

Group	*A*				*B*			
Temperature	30° C		25° C		25° C		30° C	
4-hr periods of 16-hr day	1st	2nd	3rd	4th	1st	2nd	3rd	4th
Total number of adults emerged after 5 16-hr days	33	23	112	200	152	250	14	19

TABLE 2*b*. NUMBER OF ADULTS EMERGING AT CONSTANT TEMPERATURE AFTER A 16-HR PERIODICITY OF TEMPERATURE ALTERNATION

Experimental conditions	Time of observations					
	21	1	5	9	13	17
Group *A* at 25°C for 2 days	29	32	99	150	51	35
Group *B* at 30°C for 2 days	87	137	80	57	39	49

Effect of light

In the following experiments the light source was a 60 W electric light bulb about 5 ft above the pupae. For darkness the troughs containing the pupae were covered in black cloth, when the air temperature inside the trough fell by about 0·1°C.

In these conditions, with alternate 12-hr periods of light and darkness, most of the adults emerged at about the start of the dark period, which agrees with Scott's results.

In a similar experiment, one group of pupae had the two areas of pupal cuticle that cover the developing compound eyes painted with a black stove enamel.

Their emergence rhythm was found to be similar to that of an untreated group of pupae, even when, on a repetition of the experiment, the light intensity to which both groups of pupae were exposed was reduced from about 5 to 0·08 lumens/ft².

To determine whether light or temperature was the relevant factor, one group of pupae was kept in darkness each day from 9.00 till 21.00 hr, and in light from 21.00 till 9.00 hr. During exposure to light the air temperature in the trough was 25·1°C, during the period of darkness the trough was kept in another part of the constant temperature room, where the air-temperature was 25·3°C. A control group of pupae was kept in continuous darkness and subjected to a similar variation of temperature. The control group exhibited the original endogenous rhythm, with most emergences occurring towards the evening, while the other group had a maximum emergence at the start of the dark period (Table 3). A further experiment also showed that such a rhythm associated with light and darkness is endogenous (MORIARTY, 1953).

TABLE 3. EFFECT OF ALTERNATION OF LIGHT AND DARKNESS ON THE NUMBER OF ADULTS EMERGING

Experimental conditions	Time of observations					
	13	17	21	1	5	9
Light 21–9 hr for 5 days	184	111	23	0	17	85
Continuous darkness for 5 days	68	102	127	58	24	41

To elucidate the manner in which the light influences the emergence rhythm, five groups of pupae were exposed daily to light at 21.00 hr and were returned to darkness after various multiples of 4 hr had elapsed. Observations were started after one day. The results, shown in Fig. 1, re-affirm that it is the *light variations*, and not the concomitant temperature variations, that induce an emergence rhythm; if it were only the associated temperature effects that influenced emergence the maximum rate of emergence for all five groups would have occurred at about the time of transference from light to darkness. It seems that the shorter the light period the earlier the peak of emergence, although the variation in duration of the light periods is greater than the corresponding variation of the time of the maximum rate of emergence.

For further information, pupae were subjected to single changes of light intensity. Pupae that had been exposed to varying environmental conditions for several days were placed in the constant temperature room in continuous light or darkness. When it was clear that an endogenous rhythm of emergence existed, one half of the pupae was transferred to darkness, if they had been in continuous light, or to light if in continuous darkness. In all instances the control half continued to exhibit the original endogenous rhythm. In general the results

obtained with the transferred pupae showed that transference to light delayed emergence, and transference to darkness stimulated it; the magnitude of these effects depended on what part of the daily cycle had been reached when the transference occurred. Thus, when pupae that had been in darkness were exposed to light 4–8 hr after the maximum rate of emergence had occurred the inhibitory

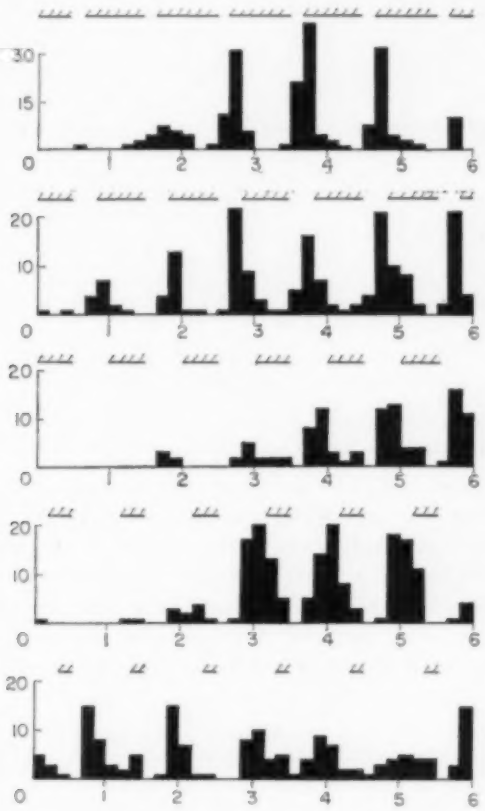


FIG. 1. Emergence of adults in alternating light and darkness.
Ordinates: number of emergences.
Abscissæ: time from start of experiment in days.
Cross-hatching indicates times of darkness.

effect of light on emergence was not very noticeable, for few emergences would be expected at this time, and the original rhythm continued, with little disturbance. However, with suitable timing relative to the endogenous peak of emergence, the single transference into either light or darkness clearly initiated a new rhythm (Tables 4 and 5).

VOL.
3
1959

TABLE 4. EFFECT OF TRANSFERENCE FROM DARKNESS TO LIGHT ON THE NUMBER OF ADULTS EMERGING

Experimental conditions	Time of observations					
	17	21	1	5	9	13
4 days' continuous darkness	80	45	17	7	21	53
Pupae transferred to light at 13.00 hr for 2 days	8	30	62	35	24	17

TABLE 5. EFFECT OF TRANSFERENCE FROM LIGHT TO DARKNESS ON THE NUMBER OF ADULTS EMERGING

Experimental conditions	Time of observations					
	9	13	17	21	1	5
3 days' continuous light	7	18	64	34	18	16
Pupae transferred to darkness at 5.00 hr for 3 days	63	35	7	5	50	97

Interaction of light and temperature

In normal conditions fall of temperature and decrease of light intensity occur at about the same time of day. Both these changes stimulate emergence.

Some pupae were kept daily from 9.00 to 21.00 hr at 25°C and from 21.00 to 9.00 hr at 30°C. They were in darkness while at the higher temperature, and in light at the lower temperature. Observations commenced after two days. The results are very similar to those obtained with pupae subjected to a similar alternation of light and darkness at a constant temperature (Table 6). On the

TABLE 6. EFFECT OF COMBINED LIGHT AND TEMPERATURE ALTERNATIONS ON THE NUMBER OF ADULTS EMERGING

Experimental conditions	Time of observations					
	13	17	21	1	5	9
4 days with light and 25°C 9–21 hr	5	31	73	90	38	7
3 days with light 5–17 hr and 25°C 9–21 hr	47	69	175	40	15	14

fifth day of observation the pupae were transferred to light and darkness 4 hr earlier, but the time of temperature change remained the same. The peak period

of emergence also came forward by about 4 hr. In both instances the temperature variations appear to have no effect on emergence except that a few emergences occurred in all periods of the day.

Relationship with general metabolism

The rate of oxygen consumption was chosen as the index of metabolic rate. Single pupae were kept at 25°C in a differential respirometer (DIXON, 1956). The position of a paraffin droplet in the connecting tube was recorded automatically at hourly intervals when a light shone for a few seconds: the paraffin droplet acted as a lens and focussed light on to a rotating sheet of bromide paper. There was no sign of a 24-hr variation in the amount of oxygen consumed: if one existed, the magnitude of the variation must have been less than 5 per cent of the total oxygen consumption.

During these experiments the oxygen tension in the reaction flask decreased. Experiments with standard Warburg manometers showed that the oxygen consumption of single pupae is unaffected by a decrease to 10 per cent oxygen. Subsequently, pupae were kept in various mixtures of nitrogen with low percentages of oxygen. No emergences were obtained in mixtures containing up to 6 per cent oxygen. With 7 per cent oxygen evidence was found of a lengthening of the periodic time, although it was not very pronounced, the peak of emergence

VOL. 3 1959

TABLE 7. EFFECT OF 7 PER CENT OXYGEN ON THE ENDOGENOUS EMERGENCE RHYTHM

Day of Experiment	Time of observations					
	13	17	21	1	5	9
			Air			
1	0	5	45	7	3	2
			7% *Oxygen*			
2	2	9	14	5	8	9
3	5	8	10	12	8	6
4	3	6	9	12	7	4
			Air			
5	12	16	29	56	23	15

TABLE 8. EFFECT OF LOW TEMPERATURE ON THE NUMBER OF ADULTS EMERGING DURING 5 DAYS

Experimental conditions	Time of observations					
	13	17	21	1	5	9
Normal	59	63	58	52	25	27
12 hr at 0·5°C	76	25	21	85	119	84

being retarded by about 4 hr after three days (Table 7). The control pupae, exposed to air, showed a normal emergence rhythm.

In a final experiment two groups of pupae were kept in varying conditions for several days, after which one group was placed at 9.00 hr in the constant temperature room at 25°C in darkness. The other group was moved at the same time, but was kept in a refrigerator at 0·5°C for 12 hr before being placed in the constant temperature room. Both groups exhibited an endogenous emergence rhythm, but they were 12 hr out of phase (Table 8.)

DISCUSSION

A fluctuating external factor cannot control an endogenous rhythm unless its variations have the same periodic time as the rhythm. SCOTT (1936) discussed such factors, and concluded that light and temperature are probably the most important. PICTET (1918) concluded, from observations on many species of Lepidoptera, that a decrease of atmospheric pressure stimulates, and an increase of atmospheric pressure retards, emergence from the pupal cuticle. No indication could be found of such an influence on emergence in *E. kühniella*.

SCOTT showed that temperature variations induced emergence rhythms, with maximum emergence occurring at that time when the temperature starts to fall. He also performed four experiments to discover whether variations of light intensity have any effect on the emergence rhythm. In the first three experiments the results were quite possibly caused by temperature changes associated with the light changes. For the last experiment the pupae were left in their cultures of flour inside tins. They were kept in a room that received strong daylight and had a sufficiently pronounced diurnal periodicity of temperature to induce an emergence rhythm in the control pupae, which were in continuous darkness. The temperature started to fall at about 15.00 hr, and most of the moths emerged from the control pupae during the period 15.00–19.00 hr. Similar results were obtained with pupae exposed to light daily from 3.00 hr till 11.00 hr; but with pupae exposed to light from 3.00 to 19.00 hr, although many adults emerged during the period 15.00–19.00 hr, maximum emergence occurred about 3 hr later than in the other two groups of pupae. SCOTT took this as proof of the inhibitory effect of light on emergence, but it was possible that the continued exposure of the pupae to light delayed the time at which the temperature started to fall. There is also the incidental difficulty, which SCOTT states, of understanding how light could affect pupae that are inside their cocoons and covered by flour.

The experiments described in this paper demonstrate the influence of light variations, independent of any associated temperature variations, on the emergence rhythm. A subsidiary reason for thinking that perhaps the associated temperature change was the real cause is the difficulty of understanding how pupae in their normal environment could perceive light. This objection is reduced since it has been shown that quite a low light intensity was sufficient to start a rhythm, even when the developing compound eyes were painted.

Workers investigating the emergence rhythm of adult insects have usually found that variations of light intensity appear to affect emergence, although sometimes associated temperature changes may be important (see PALMEN, 1955, for review of the literature). The exact mechanism, however, appears to differ in different species. BRETT (1955) found that in *Drosophila melanogaster* a single period of illumination of between 1 min and 12 hr in the larval stage is sufficient to establish an emergence rhythm, with the phases determined by the time at which the light period started. In *Ephestia kühniella* the length of the light period is important: the longer the light period, the longer the time from the start of the light period when the maximum emergence occurs (Fig. 1). This is true also of the chironomid *Pseudosmittia arenaria* (REMMERT, 1955).

In *Ephestia kühniella* it is suggested that this result is a combination of the inhibitory effect of transference to light and the stimulatory effect of transference to darkness. REMMERT found that in continuous darkness no adults of *Pseudosmittia arenaria* emerged, so the mechanism cannot be identical. BRETT (1955) found that, although continuous light does not prevent emergence of *Drosophila melanogaster*, it does upset the rhythm.

VOL. 3 1959

There is often the possibility that a rhythm said to be endogenous is actually exogenous. One can envisage a periodically varying external factor by which the periodic time of the rhythm is kept constant, but which does not determine at what time the maximal and minimal values shall occur. This possibility of dependence on a varying external factor is excluded if the periodic time of the rhythm can be altered in uniform conditions.

MICHAL (1931) states that motionless pupae of *Tenebrio molitor* have as large a daily variation of oxygen consumption as the larvae, and that it varies by about a factor of two. No such variation could be found with *Ephestia kühniella*. By keeping pupae at 0·5°C for 12 hr the emergence rhythm was delayed by a similar period (Table 8). This result, and MICHAL's, support the idea that some metabolic process is involved, although it must be independent of temperature to some extent, for transference from 25° to 30°C does not alter the rhythm (Table 1).

HARKER (1958), in a review of 24-hr rhythms throughout the animal kingdom, suggests that cells have an innate 24-hr periodicity of activity. If the emergence rhythm is controlled by such a rhythm of the nervous system, it is not surprising that no rhythm of general oxygen consumption could be found. Various mechanical analogies have been suggested. Recently BÜNNING (1958) suggested an analogy with oscillation that also allows for results obtained by the temporary subjection of organisms to temperatures in the range 0–10°C.

Acknowledgements—My thanks are due to Emeritus Professor J. W. MUNRO, in whose department this research was conducted. I would also like to thank Dr. N. WALOFF and Mr. R. G. DAVIES for their advice, and I am especially indebted to Professor O. W. RICHARDS, F.R.S., who was my supervisor during the course of this work. This work was done during the tenure of an Agricultural Research Council studentship.

REFERENCES

BREMER H. (1926) Über die tageszeitliche Konstanz im Schlupftermin der Imagines einiger Insekten und ihre experimentelle Beeinflussbarkeit. *Z. wiss. Insektbiol.* **21**, 209–216.

BRETT W. J. (1955) Persistent diurnal rhythmicity in *Drosophila* emergence. *Ann. ent. Soc. Amer.* **48**, 119–131.

BÜNNING E. (1958) Cellular clocks. *Nature, Lond.* **181**, 1169–1171.

DIXON M. (1956) *Manometric Methods*. 3rd edn. Cambridge University Press.

HARKER J. E. (1958) Diurnal rhythms in the animal kingdom. *Biol. Rev.* **33**, 1–52.

MICHAL K. (1931) Oszillationen im Sauerstoffverbrauch der Mehlwurmlarven (*Tenebrio molitor*). *Zool. Anz.* **95**, 65–75.

MORIARTY F. (1953) A study of the conditions under which the moth, *Ephestia kühniella* Zell., exhibits diurnal periodicity. Ph.D. thesis, University of London.

PALMEN E. (1955) Diel periodicity of pupal emergence in natural populations of some Chironomids (Diptera). *Ann. (bot.-zool.) Soc. zool.-bot. fenn. Vanamo* **17**, 1–30.

PARK O. (1941) Quantitative determination of rhythmicity in organisms. *Ohio J. Sci.* **41**, 39–45.

PICTET A. (1918) Les éclosions de papillons et la pression barométrique. *Bull. Soc. lépid. Genève* **4**, 67–74.

REMMERT H. (1955) Untersuchungen über das tageszeitliche gebundene Schlüpfen von *Pseudosmittia arenaria* (Dipt., Chironomidae). *Z. vergl. Physiol.* **37**, 338–354.

SCOTT W. N. (1936) An experimental analysis of the factors governing the hour of emergence of adult insects from their pupae. *Trans. R. ent. Soc. Lond.* **85**, 303–329.

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 367 *to* 377. *Pergamon Press Ltd., London. Printed in Great Britain*

STUDIES ON THE DEGENERATION OF THE FLIGHT MUSCLES OF ALATE APHIDS—II HISTOLOGY AND CONTROL OF MUSCLE BREAKDOWN

BRUCE JOHNSON

Waite Agricultural Research Institute, University of Adelaide*

(*Received* 24 *February* 1959)

Abstract—Within a few days of settling down on a host plant, alate aphids of most species undergo a change in metabolism which becomes manifest in breakdown of the flight muscles, resumption of embryo development in the ovarioles, and hypertrophy of the fat body. In this paper an account is given of the histology of flight muscle breakdown, and experimental evidence is presented which suggests that the change in metabolism in the aphid is brought about by the secretion of a hormone. Muscle breakdown and fat body hypertrophy could be initiated in aphids in response to abnormal afferent stimuli, as when aphids were tied down on inert surfaces.

VOL. 3 1959

INTRODUCTION

When alate aphids of most species settle down to feed and reproduce on a host plant they undergo a change in metabolism that results in their conversion from a migratory to a sedentary insect: their flight muscles degenerate and reproductive development proceeds. In a previous paper (Johnson, 1957) an account was given of the time of occurrence of flight muscle breakdown in alatae of a number of species of aphids in relation to reproductive development. In this paper the various histological events which occur at this time are described, and an account is given of experiments which were conducted in an attempt to determine the nature of their controlling mechanism.

MATERIALS AND METHODS

The aphids used were mostly alate alienicolae of *Acyrthosiphon pisum* Harris which were reared on young broadbean (*Vicia faba* L.) plants. A few of the observations relate to other species; where this is so it is indicated in the text.

In histological studies the aphids were fixed in Carnoy's fixative, double embedded in celloidin and paraffin and sectioned at 5μ. The sections were stained with Delafield's haematoxylin and eosin and examined under ordinary illumination and phase contrast.

In a number of experiments aphids were kept off their host plant for various periods. Aphids which are kept off their host in the light are normally very active and soon die of desiccation and exhaustion. In the present experiments they were

* Most of the work described in this paper was carried out in the Department of Zoology, University of Cambridge, England.

kept at 13°C in darkness in petri dishes containing a damp filter paper. Under these conditions they frequently survived for as long as 10 days. The aphids were sometimes found with their stylets inserted in the filter paper. Feeding however was not apparently essential for survival during this time as decapitated aphids survived for comparable periods.

While they were being operated on, the aphids were anaesthetized in a stream of carbon dioxide. Operations were done with fine needles and scalpels and the wounds were sealed with paraffin wax applied with a microcautery. Aphids are delicate soft-bodied insects and gentle handling was necessary to ensure their survival, but it was surprising what mutilations they could survive.

FLIGHT MUSCLE BREAKDOWN

The thoracic musculature of alate aphids has been described by WEBER (1930). In the mesothorax, the dorsoventral muscles, the longitudinal muscles, and some pleural muscles are well developed and are responsible for vibration of the wings during flight. Basalar and subalar muscles are absent in aphids and their place is taken by modified pleural muscles. In the metathorax, the flight muscles are not highly developed and the hindwings are coupled to and motivated by the forewings during flight.

All of the large direct and indirect flight muscles in the pterothorax were found to break down in the imago. These included the following muscles which are named according to WEBER'S (1930) classification of the muscles of the thorax of alate aphids:

Mesothorax—Dorsal longitudinal muscles 1 and 2; dorsoventral muscles 1 and 2; pleural muscles 1 to 4 (the remaining smaller pleural muscles 5 to 12 persist).

Metathorax—Dorsoventral muscles 1; pleural muscles 1.

These constitute all of the main direct and indirect flight muscles, and aphids in which these muscles have broken down appear to be incapable of vibrating their wings at all, although they are able to elevate and fold them, these movements presumably being carried out by the small pleural muscles (II pm, 10, 11, 12) which remain intact. Normally the commencement of muscle breakdown and the rate at which it proceeds are more or less uniform in the different muscles. In a number of insects, however, muscles were found in various stages of dissolution, the ventral bundles of the longitudinal muscles, I, and the dorsoventral muscles, I, usually showing less advanced breakdown than the other muscles.

The muscles do not break down at the same rate in all species of aphids. In *Aphis fabae* Scop. breakdown generally proceeds slowly, and even in insects which have been settled down on a host plant for two weeks there is frequently a large amount of muscle left. In *A. pisum*, on the other hand, very little remains of the muscles after one week.

Breakdown of the flight muscles consists of the apparently independent dissolution of the fibrils. In fixed preparations, the first evidence of breakdown of the fibrils is the appearance in them of small refractile 'droplets'. These increase

in number until they occupy the whole of the fibril which by then has become considerably swollen: they are then apparently released into the surrounding medium. The cross striations of the fibrils can still be distinguished after the appearance of the first 'droplets' in them. As autolysis proceeds the remaining fibrils become more widely separated from one another. In fixed preparations this is most apparent towards the ends of the muscle fibres, possibly because the muscle further removed from the attachments tends to become compressed during fixation. The sarcolemma or muscle sheath remains intact during and after muscle breakdown and the products of the degenerating muscles must be transported through it into the haemolymph. No evidence of the penetration of phagocytic blood cells through the muscle sheath, or of their accumulation outside the sheath, could be found.

During histolysis the muscle nuclei undergo various changes. In intact muscles they are arranged in rows between the fibrils and are small, elongated, and densely staining. As the fibrils degenerate the nuclei become scattered throughout the remaining fibrils, increase considerably in size and become more rounded. After the fibrils have disappeared the nuclei persist for some time in a pycnotic condition in the muscle sheath and sometimes become grouped into spherical bundles as their numbers are reduced.

VOL. 3 1959

After the fibrils have disappeared, the tonofibrillae by which the muscles were originally attached to the cuticle can still be distinguished in the hypodermis. As these structures are cuticula and are continuous with the cuticle their survival might be expected. As also might be expected, the tracheal system ventilating the flight muscles does not break down but persists in the thorax, and after the disappearance of the muscles it tends to resemble the 'tracheal lung' of *Nepa* which apparently originates in the same way (Hamilton, 1931).

OTHER CHANGES TAKING PLACE CONCURRENTLY WITH MUSCLE BREAKDOWN

Breakdown of the flight muscles is only one of a number of changes which take place in the aphid at this time. Other changes coincident with muscle histolysis are renewed development of the embryos, hypertrophy of the fat body, deflation of the pterostigmas, distension of the stomach and abdomen, and changes in the behaviour of the aphids (Johnson, 1957).

During the last larval instar and the long teneral period following the final ecdysis, there is extensive development of the embryos in the ovarioles of alate aphids. Embryo development characteristically ceases or is greatly reduced soon after the end of the teneral period, and until the aphid settles down to feed on a new host there is very little further change in the size or degree of differentiation of the embryos. Alatae of *A. pisum* contain about 6–8 fully developed embryos that normally increase in size after the aphid settles and before they are born, but can be born and survive without the parent aphid feeding at all after the final ecdysis. In this respect it is similar to *Macrosiphum euphobiae* (Johnson, 1957). Embryo development that takes place between settling and the initiation of flight muscle

breakdown appears to be limited to these largest embryos in the ovarioles; extensive development of the numerous smaller embryos does not begin until about the time the flight muscles begin to degenerate.

In aphids with intact flight muscles the fat body in the thorax is compressed into the relatively small spaces between the flight muscles; after the muscles have degenerated the fat body fills a far greater part of the thorax. This increase in volume of the fat body is due entirely to hypertrophy of the cells and *not* to an increase in cell number. In the abdomen there is a similar hypertrophy of the fat body and this is most apparent in the peripheral layer. The fat body cells subsequently become heavily laden with fat.

Before settling on a host, the pterostigmas of young alate aphids are inflated with haemolymph and form roughly cylindrical structures situated about two thirds of the way along the forewing and extending in some species for about a quarter of its total length. When aphids are feeding, the stigmas become partly deflated, but as long as the aphids are still able to fly the stigmas soon become reinflated whenever the aphids are disturbed and induced to withdraw their stylets. Once the flight muscles have begun to break down and the aphids have lost the ability to fly, the stigmas no longer become fully inflated when the aphids are disturbed, and in aphids in which the flight muscles are very reduced the stigmas frequently become completely flattened and dry and the wings become more brittle suggesting that blood circulation to the wings has been interfered with.

The surface of the cuticle of young alate aphids before settling down on a host plant is clean and shiny: in old aphids whether or not the muscles have begun to break down the entire cuticle becomes covered with a waxy bloom so that it loses its shiny appearance and becomes dull. This change is most marked on the head and the dorsum of the mesothorax.

Within a few days of settling the forepart of the midgut shows a considerable increase in size, the length and diameter frequently increasing to twice their size before settling. As the stomach becomes larger it is pushed up farther into the thorax and comes to occupy much of the space left by the degenerating muscles.

Aphids in which flight muscle histolysis has begun are readily distinguishable externally from aphids with intact muscles by the condition of their wings and cuticle and the size and shape of the abdomen. As embryo development and fat body hypertrophy proceed the abdomen becomes greatly distended resembling somewhat the shape assumed by aphids parasitized by *Aphidius* wasps. This shape is retained for the rest of the lives of the aphids so long as they continue to feed and although later in their lives the abdomen contains very few embryos and is mainly filled with haemolymph.

CONTROL OF MUSCLE BREAKDOWN

It was noticed that the flight muscles normally only broke down in those aphids which settled down and remained feeding and reproducing on host plants: aphids which were kept off host plants remained alive for up to 10 days but whether or not they had previously undertaken any flight their muscles did not break down.

Neither did they break down in aphids which after reaching maturity were left for 3–4 days on host plants in darkness. This was due to their failure to settle down on the host to feed and reproduce, that is, to show the settling response (JOHNSON, 1958). As has been mentioned (*op. cit.*), this kind of behaviour of remaining on host plants but not 'accepting' them in the sense of showing some further manifestation of the settling response sometimes also occurs in aphids which are kept in apparently suitable flight conditions. In the following experiments the aphids were either flown for 20 sec or given a knockdown dose of carbon dioxide and they were then released on bean seedlings; this was effective in releasing the full settling response and the aphids settled down and remained on the plants.

In alatae of *A. pisum*, which were kept in a laboratory at an average temperature of about 15°C, muscle breakdown was first apparent on the second day after the aphids had settled on their host. To find what length of time on the host was necessary before muscle breakdown was initiated, batches of aphids were removed at intervals after settling and were then kept off the host in petri dishes. It was found that aphids removed within about 24 hr nearly all retained intact muscles whereas in most of those removed after this time, the muscles went on to break down. The results of this experiment are given in Table 1. The aphids in which

VOL. 3 1959

TABLE 1. NUMBERS OF APHIDS WHICH DEVELOPED MUSCLE BREAKDOWN WITHIN FOUR DAYS OF REMOVAL FROM HOST

Treatment	Time spent on host			
	18 hr	24 hr	36 hr	48 hr
Intact	2/15	10/15	14/15	15/15
Decapitated	0/15	8/12	9/10	15/15

the muscles broke down all had fully distended abdomens when they were removed from the host, the others had enlarged but not fully distended abdomens. It is possible that the latter aphids had remained 'sitting' on the plants for some time before settling down to feed and were thus physiologically behind the other aphids which apparently settled down to feed straight away and went on to break down their muscles in the minimum time. In a further experiment the aphids were decapitated at intervals after settling and essentially the same results were obtained: aphids decapitated before 24–36 hr retained intact muscles, in aphids decapitated later the muscles broke down. Although muscle histolysis continued to some extent in the decapitated aphids, it did not proceed as rapidly as in intact aphids, and in none of the aphids which had been removed from the host plant was fat body hypertrophy as conspicuous as in aphids which were kept on host plants throughout.

As the change in metabolism of the aphid is of a general systemic nature involving a number of organs, it seemed possible that a common stimulus may have

been concerned and that this may have been hormonal. A number of attempts were made to investigate this possibility. Implants of various organs such as the brain and corpus allatum were made from aphids which had been settled down for 2–3 days into young decapitated aphids, but no positive results were obtained and the mortality rate was high enough to discourage further attempts along these lines.

Another approach was made by transfusing young decapitated aphids with the blood of aphids in which the muscles were beginning to degenerate. This was achieved by means of parabiosis. Young adult aphids were decapitated and joined in parabiosis to young intact alatae and apterae which had been settled down on host plants for 2–3 days. The neck of the decapitated insect was inserted in a small hole cut in the dorsal surface of the abdomen of the intact aphid: the decapitated aphid was then carried about on the back of the intact aphid and its legs were stuck together with wax in order not to interfere with its 'host's' movements. A number of aphids from which the head and prothorax were removed were treated in the same way. After the operation the aphids were kept on host plants and the intact aphids invariably settled down to feed and reproduce. The genital aperture of the decapitated aphids was occluded with a drop of wax, as it has been found that decapitation of aphids releases the larviposition response (JOHNSON, unpublished) and it was necessary to prevent them from giving birth to young to make it possible to assess whether or not they were showing any embryo development as a result of the treatment.

After 4–6 days the aphids were dissected or sectioned and the condition of their flight muscles and whether or not there had been extensive embryo development were recorded. The results of the experiments are given in Table 2.

TABLE 2. RESULTS OF PARABIOSIS EXPERIMENTS

Treatment	Muscle breakdown and embryo development	Muscle breakdown and no embryo development	No muscle breakdown or embryo development
Decapitated and joined to alatae	1	8	8
Decapitated and joined to apterae	6	32	7
Head and prothorax extirpated and mesothorax joined to apterae	2	6	0
Decapitated aphids joined to one another	0	0	10
Frons removed and aphid joined to apterae	2	21	0

VOL. 3 1959

FIG. 1. Section of thorax of *Acyrthosiphon pisum* which had been settled on a host plant for three days. Note the large round nuclei and separated fibrils of the dorsoventral muscle which is undergoing histolysis, and the intact small pleural muscle. (× 250.)

FIG. 2. End of dorsoventral muscle of *A. pisum* showing further stage in degeneration of the muscle: only scattered fibrils remain. (× 420.)

FIG. 3. Section of thorax of an aphid which had been decapitated and joined in parabiosis to an intact aphid for six days. One dorsoventral muscle only is showing extensive degeneration. (× 100.)

FIG. 4. Detail of dorsoventral muscle shown in Fig. 3. The muscle has lost its original structure and nuclei and fragments of fibrils lie in an amorphous basophilic matrix. (× 950.)

In most of the decapitated aphids there was clear evidence of muscle breakdown but only in a few was there extensive muscle breakdown and embryo development. In the aphids in which there was embryo development, muscle breakdown was comparable in extent to that in intact aphids but it differed in its histological details. Instead of the fibrils always breaking down and disappearing entirely, the degenerative products of the muscles remained inside the sarcolemma, persisting as basophilic material containing vacuoles, nuclei and sometimes fragments of bundles of fibrils. In the aphids in which there was no embryo development muscle breakdown was more irregular in that frequently only a few muscle bundles, most often the dorsoventral muscles II, broke down, the rest remaining quite intact.

It was shown above that muscle breakdown did not proceed in decapitated aphids unless it had been initiated before decapitation. Yet the muscles did break down when decapitated aphids were transfused with the blood of intact aphids. As muscle breakdown induced in this way was frequently incomplete it was possible that its occurrence may have been due to an incompatibility between the blood of the two insects. To test this, decapitated insects were joined to one another by their severed necks. Of five pairs which survived for six days none showed any degree of muscle breakdown.

VOL.
3
1959

It seems therefore that some factor present in the blood of the aphids which had settled down to feed transfused from the intact to the decapitated aphids and caused their muscles to break down. The fact that only in a few cases was there extensive embryo development and complete muscle breakdown could have been due to there generally being an inadequate flow of blood between the two insects for these processes in the decapitated aphids to be fully controlled.

In an attempt to find whether breakdown of the muscles depended in any way on embryo development or whether these processes were independent of one another a number of aphids were castrated by removing as much as possible of their ovaries. This operation is rather drastic in these insects as the ovaries fill most of the abdominal cavity and it was impossible to remove them without removing part of the alimentary canal as well. Five aphids which were operated on survived for four days and by this time their flight muscles were breaking down and the fat body was showing hypertrophy. Each of them however contained some remains of the ovaries although these were very small and it is unlikely that they would have been significant in affecting the flight muscles or fat body.

It is thought that the corpus allatum hormone in insects is concerned in protein metabolism (WIGGLESWORTH, 1954). As the various processes which take place at this time in aphids involve protein metabolism it was suspected that the corpus allatum hormone may be involved here. The corpus allatum in aphids is a large conspicuous body and it was examined in dissections and serial sections of ten aphids before and ten aphids five days after settling on host plants. No conspicuous changes in its size or appearance were observed.

MUSCLE BREAKDOWN IN RESPONSE TO ABNORMAL AFFERENT STIMULI

A number of aphids were joined in parabiosis to intact aphids after removing only the frons rather than the whole head and it was invariably found that in all of them the flight muscles broke down regularly and rapidly, but again only in a few was there any apparent embryo development (Table 2). Because muscle breakdown in these insects was so uniform, and as they still retained their own brain, it seemed probable that the stimulus responsible for its initiation may have been originating within the insect itself. In an attempt to determine whether or not a wound hormone was implicated, aphids were injured in various ways and then kept in darkness in petri dishes for four to five days. Some injuries resulted in flight muscle breakdown: among them were severing the abdomen and then grafting it on again, and cutting off all or most of the legs and antennae: cutting off only a few legs had no effect.

In one experiment the filter paper in the petri dish in which the aphids were kept was too wet and the wings of some of the aphids became stuck to it by surface tension. All of these aphids were found to have degenerating flight muscles. Thus it appeared that the effect may not be a consequence of physical injury sustained by the aphids, but rather a response to the effect of the injury in interfering with their normal behaviour. Further evidence in support of this was obtained by tying aphids down on pieces of cork with pins and nylon thread, or on filter paper by sticking their tarsi down with wax or glue. Within four days all the aphids treated in this way showed muscle breakdown and hypertrophy of the fat body although there was no apparent embryo development. The filter paper to which the aphids were stuck was kept moist, and the aphids inserted their stylets in it and imbibed some water as indicated by the fact that their rectum became distended and when neutral red was put in the water their midgut became strongly stained. This feeding, however, was not essential for the initiation of muscle breakdown as the muscles also broke down in aphids kept on dry filter paper and cork but in a humid atmosphere to prevent them from desiccating. Most of the fully developed embryos they contained were deposited by the aphids when they were tied down. If they were unable to imbibe water their abdomen then became contracted.

In these experiments the aphids sustained no apparent physical injury and they remained healthy. Some of them were released after they had been tied down for five days and put on host plants and they settled down to feed and reproduce just as normal aphids.

DISCUSSION

Breakdown of the flight muscles is one of a number of changes which take place in alate aphids after they settle down on a host plant. Attention has been directed mainly on flight muscle breakdown because it is the most conspicuous of these changes and the condition of the flight muscles can be used as an indicator of whether or not the general change in metabolism of the aphid has been initiated. The other principal processes coincident with muscle histolysis are hypertrophy of

the fat body and renewed embryo development. As a result of this change in metabolism the alate aphid is converted from a migratory insect specially adapted for dispersal in possessing wings and powerful flight muscles to a sedentary insect with more the characteristics of apterae in that its energies become diverted wholly towards reproduction.

The fact that the changes that take place involve a number of organs suggests that the various processes may either be controlled by a common humoral factor, or they may be causally related. The evidence presented above suggests that they are hormonally controlled although whether or not the same factor is implicated in all the changes is not known. The fact that removal of the ovaries did not interfere with muscle breakdown suggests that muscle breakdown occurs independently of embryo development. It is not possible to say to what extent, if at all, embryo development in aphids is hormonally controlled. As aphids normally spend their whole time with their stylets inserted in a host plant and for most of the time are actively imbibing plant juices, it might be expected that the nutrient supply obtained from continuous feeding is a necessary prerequisite to extensive embryo development. When aphids were tied down on wet filter paper they inserted their stylets in the paper and imbibed water, and at this time they contained copious stores of glycogen and fat, and from the breakdown of the flight muscles they had abundant nitrogen; but there was no extensive embryo development under these conditions. This could be because either a hormone was being withheld or the developing embryos require some specific nutrients that are only present in adequate amounts when the aphids are imbibing plant sap. As aphids which are tied down do apparently secrete a hormone which initiates flight muscle histolysis and fat body hypertrophy, it seems unlikely that extensive embryo development was prevented by the lack of another hormone which was not secreted but rather that the cause of the failure of the embryos to develop under these conditions was lack of adequate specific nutrients. Parabiosis experiments can throw no light on this question, as where there is a flow of blood between two insects this can equally well carry nutrients as hormones from one insect to the other.

In a number of insects it has been shown that during development certain muscles break down while others remain intact. In experiments carried out by FINLAYSON (1956) on Saturniids, the abdominal muscles which normally only break down in the adult insect after emergence were caused to break down in the pupa by cutting their nervous connexions or by removing the ganglia from the segment containing the muscles or from the preceding segment. FINLAYSON suggested that withdrawal of neural influence from the muscles and hormone balance both probably played a part in muscle histolysis. WIGGLESWORTH (1956), on the other hand, found that breakdown of the intersegmental muscles of *Rhodnius* was independent of nerve supply and suggested that it is controlled by hormonal means. The above experiments on aphids suggest that the humoral environment is important in muscle breakdown, and if the nervous system plays a part in the aphids in the way suggested by FINLAYSON then decapitation of the aphid is

VOL. 3 1959

apparently effective in interfering with the neural influence on the flight muscles. Why other muscles do not degenerate when the insects are decapitated and supplied by parabiosis with the appropriate humoral environment is not known: there may well be some intrinsic differences in the muscles themselves.

In a recent study of the behavioural responses of alate aphids it was suggested (JOHNSON, 1958) that their behaviour is dominated by two general, mutually exclusive responses: the locomotor response and the settling response. Aphids showing the locomotor response are unresponsive to their host plants and make persistent attempts to engage in locomotory activity; the settling response includes alighting, wandering and probing, feeding and reproducing, and the initiation of flight muscle histolysis, this being the fullest degree of the settling response with the irrevocable reversion of the aphid from one with a locomotory potential to one which is inevitably more sedentary and vegetative in the sense of KENNEDY (1956). It was found that a weak settling response consisting of wandering and probing behaviour could be brought about by artificially terminating the flight of aphids after they had flown for a minimum of about 20 sec. It is interesting that in the present experiments a further manifestation of the settling response, the initiation of muscle breakdown, was also brought about by artificial methods which bore some resemblance to those which were effective at the lower level. In both cases the locomotory activity of the insects was interfered with: in the first case only momentarily, in the second case a more prolonged restraint on their locomotion was applied and this had a much more profound effect on the aphid although in the same direction, of causing them to show manifestations of the settling response, in this case larviposition and breakdown of the flight muscles.

Another instance in which interference with the locomotor apparatus of an insect has apparently led to secretion of a hormone was reported by NORRIS (1954), who found that individuals of *Schistocerca* from which legs had been amputated became sexually mature before intact individuals. NORRIS suggested that this effect may be accounted for in terms of a wound hormone being produced, but the fact that injuries were equally effective when they were inflicted before the adult stage was reached suggests that it might well have been due to the deformity caused by the injury, and that this was acting in the same way as interfering with the locomotory activity of aphids.

STILES (1938, 1939) used the amount of flight muscle present in the thorax of intermediate aphids as a factor, along with wing length and other characters in an analysis which he claimed lent support to the 'time of determination' theory of form determination. Had he taken all newly moulted adult aphids before muscle breakdown had been initiated he may have been able to use muscle amount as a factor in his studies, but he did not do this as is indicated by his illustrations of the thoraxes of two aphids showing the extremes of the amounts of flight muscle present: one aphid has intact muscles, the other has none. In the aphid with intact muscles, the alimentary canal and fat body are characteristic of aphids which have not yet settled down on a host or have only recently done so; in the aphid with no muscles the midgut is distended and the fat body cells are hypertrophied,

indicating that this was an aphid which had been settled for some time. STILES' conclusions were thus based on the false assumption that the wing muscle condition of the insects he studied was solely a manifestation of their intermediacy, but as in fact he was studying adult aphids of different ages and therefore showing different degrees of postmetamorphic flight muscle degeneration, as well perhaps as differences in extent of development of the flight muscles, they must now be regarded as untenable.

Acknowledgements—Most of the work described in this paper was done in the Department of Entomology, Cambridge University, England, while the author held an Australian Canteen Trust Fund Scholarship. I am indebted to Professor V. B. WIGGLESWORTH for making space and facilities available.

REFERENCES

FINLAYSON L. H. (1956) Normal and induced degeneration of abdominal muscles during metamorphosis in the Lepidoptera. *Quart. J. micr. Sci.* **97**, 215–233.

HAMILTON M. A. (1931) The morphology of the water scorpion, *Nepa cinerea* Linn. (Rhynchota, Heteroptera). *Proc. Zool. Soc. Lond.* **1931**, 1067–1136.

JOHNSON B. (1957) Studies on the degeneration of the flight muscles of alate aphids—I. A comparative study of the occurrence of muscle breakdown in relation to reproduction in several species. *J. Ins. Physiol.* **1**, 248–256.

JOHNSON B. (1958) Factors affecting the locomotor and settling responses of alate aphids. *Animal Behaviour* **6**, 9–26.

KENNEDY J. S. (1956) Phase transformation in locust biology. *Biol. Rev.* **31**, 349–370.

NORRIS M. J. (1954) Sexual maturation in the desert locust with special reference to the effects of grouping. *Anti-locust Bulletin* **18**. London. 43 pp.

STILES K. A. (1938) Intermediate winged aphids and the time-of-determination theory. *Biol. Bull., Woods Hole* **74**, 430–460.

STILES K. A. (1939) The time of embryonic determination of sensoria and antennal color and their relation to the determination of wings, ocelli and wing muscles in aphids. *Biol. Bull., Woods Hole* **76**, 442–447.

WEBER H. (1930) *Biologie der Hemipteren*. Berlin.

WIGGLESWORTH V. B. (1954) *The Physiology of Insect Metamorphosis*. Cambridge. 149 pp.

WIGGLESWORTH V. B. (1956) Formation and involution of striated muscle fibres during the growth and moulting cycles of *Rhodnius prolixus* (Hemiptera). *Quart. J. micr. Sci.* **97**, 465–480.

VOL. 3 1959

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 378 *to* 394. *Pergamon Press Ltd., London. Printed in Great Britain*

THE NEUROMUSCULAR MECHANISM OF AN INSECT SPIRACULAR MUSCLE

G. HOYLE

Zoology Department, University of Glasgow, Scotland

(*Received* 28 *February* 1959)

Abstract—The second thoracic spiracle of the locust *Schistocerca gregaria* is closed by the action of a small muscle which receives two motor axons from a branch of the median nerve of the mesothoracic ganglion. There are two groups of muscle fibres, an outer layer composed of thicker, uniform fibres, and an inner core of thin, irregularly shaped ones. Each of the motor axons gives an electrical response in each of the muscle fibres, both of which are in the nature of end-plate potentials, although one is always larger than the other. The larger response is attributed to a 'fast' axon and leads to a brisk twitch. The smaller response is attributed to a 'slow' axon but also leads to a twitch, though only ½ the magnitude of the other. In the intact animal the movements of the spiracular valves are regulated principally by the discharge of the fast axon. This terminates abruptly at intervals, permitting the muscle to relax, when the elastic forces in the hinges of the valves cause them to open. The durations of opening and closing are determined by nervous influences arising mainly in the abdomen. There is no evidence for a peripherally located sensory-motor nervous system affecting the functioning of the spiracular closing muscle.

INTRODUCTION

WHEN the respiratory gas exchange of insects was measured by continuous recording over long periods of time (PUNT, 1950), it was found that in pupae and hibernating insects carbon dioxide was not released continuously, but periodically, at very long intervals. Subsequent research has demonstrated that the controlling elements are the spiracular valves (SCHNEIDERMAN, 1956). These stay closed, or very nearly closed, in the intervals between carbon dioxide release, during which carbon dioxide accumulates in the tracheae and haemolymph. When the valves open, the carbon dioxide is quickly lost and the cycle is resumed. An outline theory of the chemical mechanisms involved has been proposed by BUCK (1958).

These events in the carbon dioxide release mechanism have focused attention on the physiology of the muscles which control the closing of the spiracular valves. They must be virtually indefatigable and controlled by possibly a unique mechanism. CASE (1957) has shown that the closer muscles of the spiracles of *Periplaneta americana* and *Blaberus craniifer* are innervated by two efferent axons which originate in the median (unpaired) nerves of the same segment as that on which the spiracle is situated. When impulses pass down these two axons the muscles contract. Following section of the nerves the muscles were found to retain their capacity for responding to directly applied electric stimulation, for

three months. After denervation for several days, spontaneous muscular contractions, in which groups of muscle fibres contracted in synchrony, set in. They were referred to as fasciculations. Several problems as to the nature of events in the normal control mechanism of the spiracles are raised by these observations.

Interest in the problem was heightened by the findings of BECKEL and SCHNEIDERMAN (1957) on an isolated spiracle-closer muscle preparation from the pupa of the moth *Hyalophora cecropia*. In this, the closer muscle remains contracted, showing a continuous stream of action potentials at a rate of 12–15 sec, keeping the spiracular valves closed continually. Bubbling carbon dioxide into the bathing fluid, however, caused the muscle to relax and the valves to open. The mechanism of action of the gas was not investigated.

The present work was undertaken in order to obtain a preparation in which the details of normal neuromuscular transmission in a spiracular closing muscle could be investigated. A suitable preparation was obtained using the second thoracic spiracle of the locust *Schistocerca gregaria*. The preparation has also been used to study the mode of action of carbon dioxide, and these results will be presented in a subsequent paper.

VOL. 3 1959

THE SPIRACULAR APPARATUS

Preparations can be obtained from either the second thoracic or the first abdominal spiracle. The latter is supplied by two muscles, the former by only one, a closer. The second thoracic spiracle provides extremely suitable material for the various manipulations required, and the following account refers to it exclusively. An account of the anatomy of the spiracular apparatus will be briefly reported here for convenience. Additional information has been obtained by MILLER (1959) in independent studies.

The valves consist of two semi-circular cups, the posterior one having an extended lip which overlaps the anterior one. Each is attached to a thin strip of heavily-sclerotized cuticle on its outer margin, which forms a stiff hinge. The posterior one is shaped in an acute V, permitting a greater degree of movement than the flatter and thicker anterior one. The inner cuticular layers are non-sclerotized. They are particularly thick at the base of the spiracle, forming a pad which resists the compression imposed by the closing of the valves.

A tubular invagination originating in the mid-line, at the extreme ventral tips of the valves, passes through this pad. It enlarges as it continues, still hollow in section, to form the apodeme of the closer muscle (Fig. 1).

The first muscle fibres to be inserted on the apodeme enlarge to 30–65 μ, continuing as an outer ring of some 20 fibres (Fig. 2). The core of the muscle, consisting of fibres inserted farther along the narrowing apodeme, is composed of about 45–50 much smaller fibres, rather irregular in shape. In between these central fibres there are many tracheae. Although the general appearance of a transverse section through the muscle under low power gives the impression of there being a division of the muscle into two parts, the detailed histology of the small and large fibres is not dissimilar. Both are composed of similarly-staining,

very fine fibrils. In longitudinal sections, however, a difference is apparent. The sarcomere thickness (inter Z-membrane distance) is greater in the inner fibres, being of the order of 6–7 μ, reaching 9 μ maximum, in the unstretched muscle, compared with only 3–6 μ for the outer fibres. The average thickness of the inner fibres is in the region of 10–15 μ.

In the physiological experiments to be described no obvious distinction was encountered between inner and outer fibres. It may be that the inner fibres remain undeveloped through there being too many of them to fit into the small volume of space available. An insect ordinary skeletal muscle of comparable diameter would have only a few, large, muscle fibres in the core, of similar diameter to the outer ones. The difference in the sarcomere length of the fibres of the spiracular closing muscle should lead to appreciable differences in the speeds of contraction in the two parts of the muscle, since fibres with close spacing of the Z-membranes can be expected to contract much more quickly than those with a wide separation, other factors being equal. It may be that the outer fibres are principally responsible for the rapid closure of the valves. The inner fibres may be more suited to maintaining prolonged contraction without fatigue. There would thus be very roughly a division into phasic and tonic parts in the same muscle. If this could be substantiated, the interest of the spiracular muscle would be further increased.

METHODS

The whole spiracular apparatus, valves, closer muscle, and a few millimetres of nerve can be completely isolated. But so small a preparation is not easy to handle and the muscle deteriorates rapidly. This may be due to the damage done to the tracheal supply, which appears to be the only route by which free respiratory exchange can take place, tracheolated membranes acting as a barrier to direct gas exchange across the outer surfaces. By leaving the spiracle *in situ* it is possible to obtain a very similar preparation, with the tracheal supply intact.

The head, prothoracic segment, and pronotum are first removed. The abdomen is then severed from the remains of the thorax, except in experiments in which the influence of the abdomen on the 'spontaneous' rhythm of the spiracular system is to be investigated. The portion of gut in the thorax is then pulled out and the roof of the thorax, the wings, and the wing-muscles are severed by cutting along the thoracic walls at a level just above the second pair of spiracles. At this stage the 'preparation' is partly embedded, ventral surface down, in a support shaped from modelling clay, using the legs as stays. The nerves supplying the legs are cut so that no contraction of the leg muscles will occur to cause the thorax to move. A small cavity is left in the clay in the region of the spiracle to be investigated. The whole is mounted on a mechanical stage.

Attention is now turned to the mesothoracic ganglion, which is exposed from the dorsal surface. It is from this surface that the unpaired, median nerve which contains the motor axons of the closer muscles of the second thoracic spiracles is located. The nerve issues between a pair of ventral longitudinal muscles attached

VOL.
3
1959

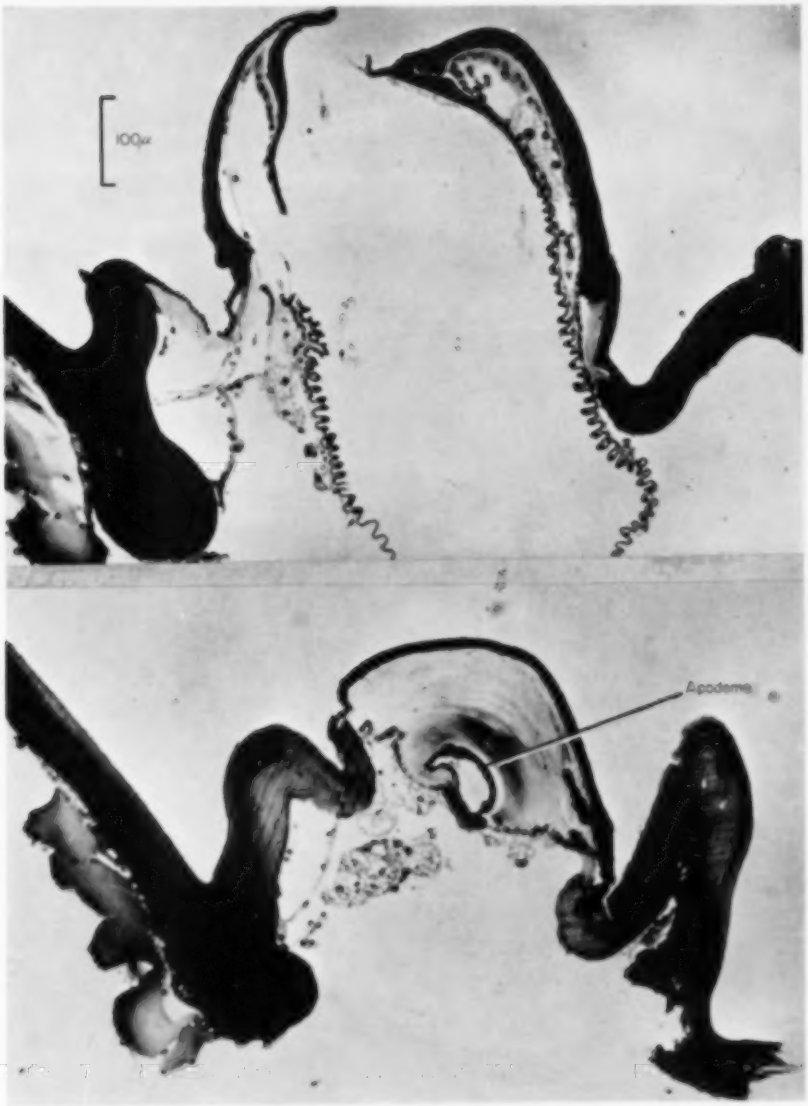


FIG. 1. Structure of the spiracle. (a) T.S. whole spiracle just below the mid-line. The lip on the left-hand side of the photomicrograph is the more mobile posterior one. Note the V-shaped region of thin sclerotized cuticle which forms its hinge; underlying non-sclerotized cuticle. (b) T.S. below extreme base of lips of second spiracle. The tubular structure is the apodeme of the closer muscle. The origins of a few of the muscle fibres of the outer ring can be seen vertically.

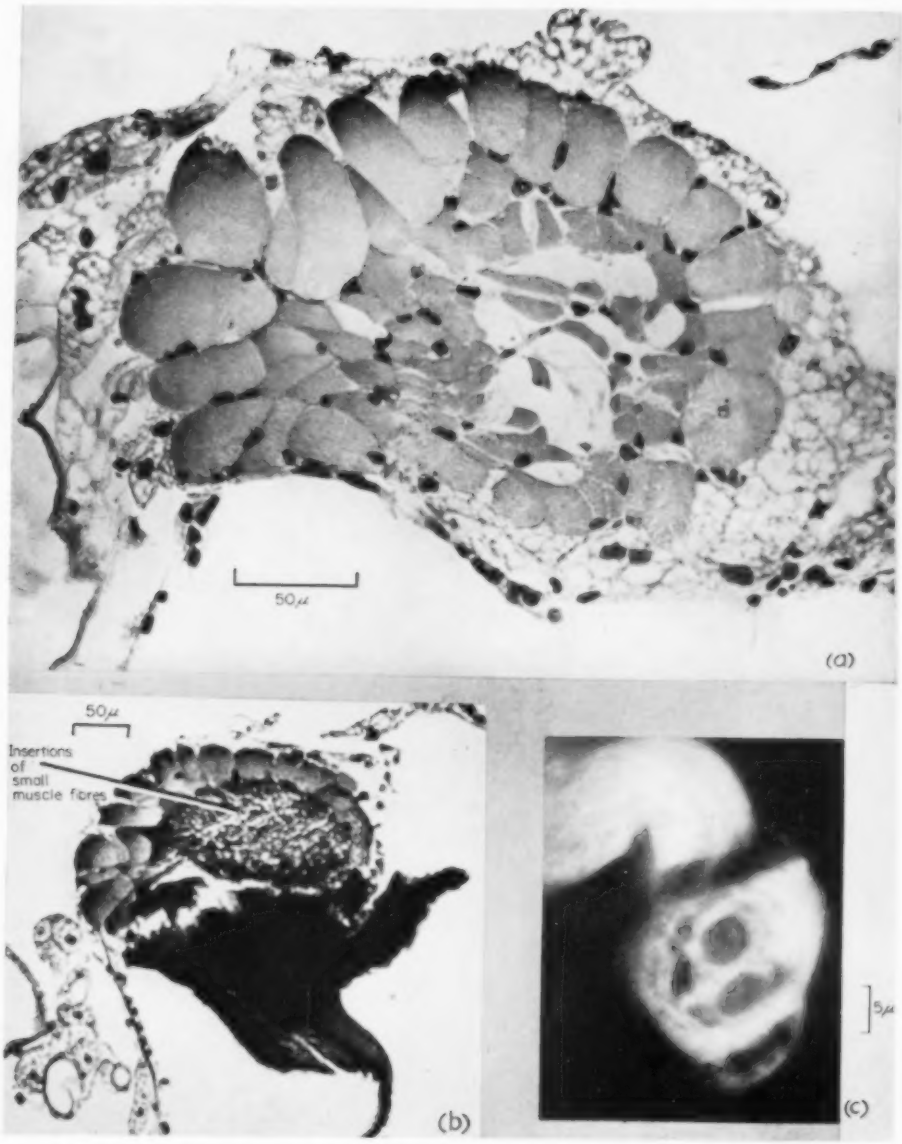


FIG. 2. Histology of the closer muscle. (a) T.S. spiracular closing muscle showing outer layer of large muscle fibres and inner layer of small, irregularly-shaped ones. (b) Section through the closer muscle in the region of its ventral insertion. The central, darkly-stained mass is composed of the attachments of the small muscle fibres. (c) Section through the motor nerve at its point of entry into the muscle. Note the two axons, 'slow' and 'fast'.

to spines which project between the nervous connectives running from the second to the first and third thoracic ganglia (Fig. 3). It is rather difficult to locate the median nerve, since it divides into the two spiracular nerves after emerging from between the muscles and these nerves then immediately pass into a diaphragm where they are almost invisible. However, by pulling apart the two muscles the median nerve can usually be located and crushed or severed close to the ganglion. Having located the spiracular nerves, one of them is freed for a distance of about 2 mm, and paired, hooked, silver electrodes are placed on it.

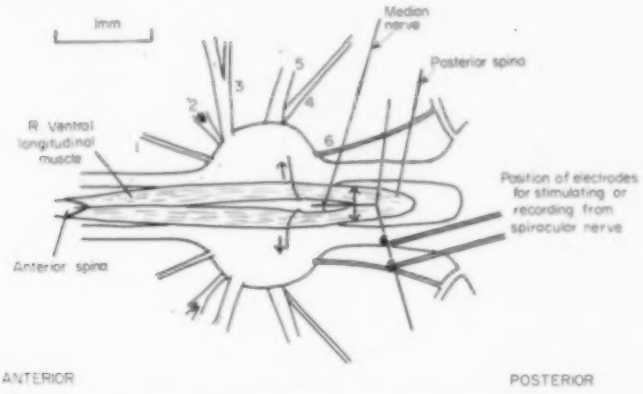


FIG. 3. Dorsal view of mesothoracic ganglion showing the position of emergence of the median (unpaired) nerve, its division into the left and right spiracular nerves, and the position in which the stimulating electrodes were placed.

One side of the animal is now illuminated, showing the spiracular valves. The posterior lip overhangs the anterior one (Fig. 4a). An incision is now made in the cuticle above the spiracle and continued along the broken line indicated in Fig. 4(b). This frees a rectangular flap of cuticle in which lies the spiracle and the spiracular closing muscle. The latter is attached ventrally to an intucked peg of cuticle and dorsally to the short apodeme which extends into the bases of the two valves. By gently pulling the flap outwards, the inside of the spiracle and the closer muscle are exposed. The tracheal trunk is severed at its junction with the spiracular lips and very carefully lifted back into its natural position. In this way it can be kept open and air, oxygen or gas mixtures delivered into it by a finely-drawn pipette.

The tracheal trunk gives off a small branch to a tiny air-sac anteriorly. This air-sac in turn supplies the closer muscle. In a good preparation the sac can be visibly inflated or deflated from the inserted pipette.

The outer (dorsal) edge of the flap is now embedded in the clay, and the small chamber surrounding the muscle filled with locust saline (HOYLE, 1953 variety). The saline is brought into contact through a channel with a large volume of similar saline in the dish containing the preparation.

VOL. 3 1959

To effect mechanical registration of the movements of the spiracle, a gramophone crystal pick-up cartridge was used. A special needle was constructed from an entomological pin by tapering it to a fine, sharp point and bending it into a 'trailer' shape. The cartridge was mounted on a heavy, adjustable stand and lowered until the tip of the needle just pierced, as far as could be judged, the

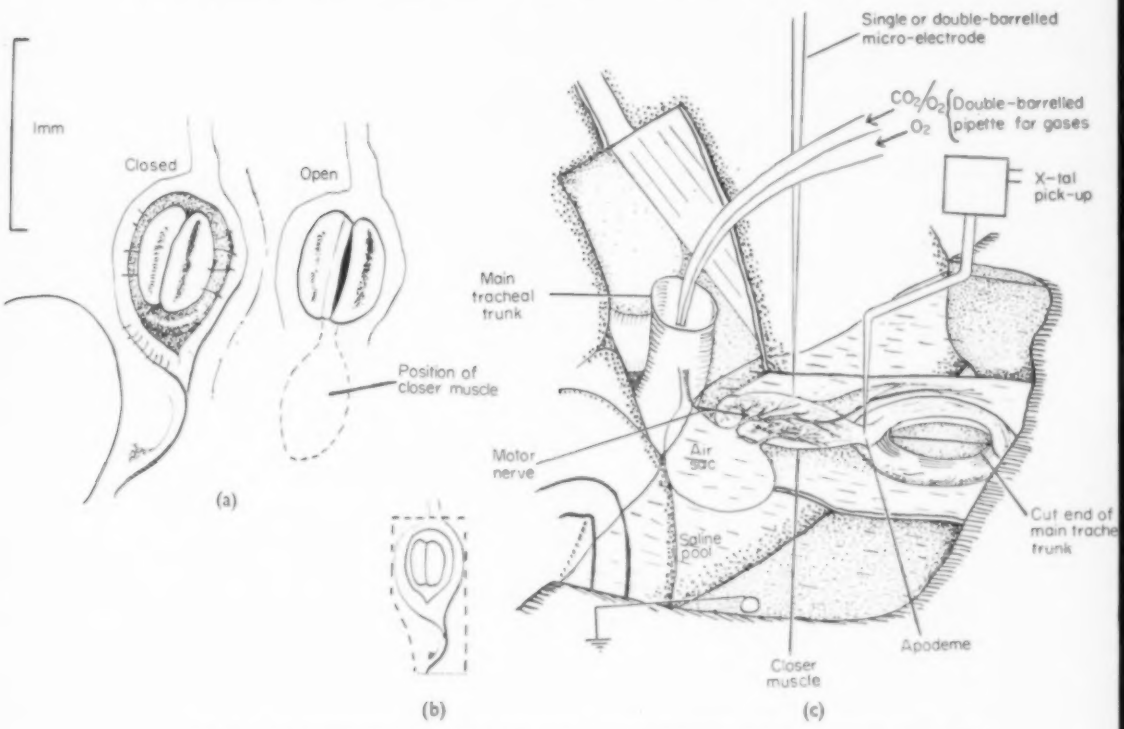


FIG. 4. The preparation. (a) The left metathoracic (second) spiracle viewed externally in the closed position (left) and the open position (right), the latter showing the position of the closer muscle (broken line). (b) The line of cutting round the spiracle, indicated by a broken line. The piece of cuticle containing the spiracle is bent outwards along the dotted line. (c) The completed preparation, external part.

apodeme. The tubular apodeme is strong enough to stand up to prolonged, vigorous contraction of the muscle with the needle inserted. A tension equal to that of the twitch of the closer muscle (about 0·1 g) moves the tip of the needle through only a few microns, so the recording situation is almost ideally isometric. The magnitude of the potentials developed in the crystal depends on the force and speed of the contraction.

With the arrangements described above completed, the final manipulation is accomplished. This consists of manœuvring a 3 M–KCl-filled glass capillary microelectrode, single or double barrelled, into position, ready to be inserted

into single muscle fibres of the closer muscle. The muscle was illuminated by an intense direct beam of light. The experiments were all done at room temperature (19–20°C, thermostatically-controlled room).

The tracheal supply of the muscle is extremely rich, the density of tracheoles being greater than in any other insect muscle which I have encountered. This makes for difficulty in inserting the electrode randomly, since it frequently encounters an 'infinite resistance' during penetration, presumably as the result of entering a tracheal tube. The electrical potentials were displayed simultaneously on a cathode ray oscilloscope, using direct-coupled amplification, and on a pen recorder, using also direct-coupling, or sometimes a condenser-coupled amplifier with a time-constant of 1 sec.

RESULTS

The muscle is innervated only from the median nerve, which supplies it with two axons, one 7 μ and the other 6 μ in diameter across the major axis, measured at the point of entry into the muscle. Both axons discharge impulses rather irregularly and the two streams are never synchronous. Sometimes it is possible to discern the individual responses due to the motor axons by recording from single muscle fibres when the rate of discharge is low (cf. Figs. 7a, b). Responses to each axon were observed in all the muscle fibres penetrated.

VOL. 3 1959

It is sometimes impossible to obtain controlled stimulation of the two axons separately since they have similar thresholds and only slightly different conduction velocities, but one axon, probably the larger one, usually has a slightly lower threshold and faster conduction velocity than the other. But the second axon can only with difficulty be stimulated alone, using tricks such as varying pulse length with the anode distal, to block the faster-travelling impulse (technique of KUFFLER and VAUGHAN WILLIAMS, 1953). The ordinary skeletal muscles of the legs of locusts are also supplied by two motor axons (HOYLE, 1955). The axons differ functionally, one is a 'fast' axon causing a quick, powerful contraction of the muscle when stimulated with a single shock, the other is a 'slow' axon which gives much weaker mechanical responses, requiring repetitive excitation of the nerve for their elicitation, and involving extensive facilitation and summation.

In the present case, the two axons each give rise to a small twitch when stimulated with a single shock. The isometric tensions developed in the two responses do not differ appreciably in their time-courses, as far as can be told from the records obtained using the crystal type of transducer. We may provisionally regard the smaller response as being similar to the other, differing principally in the magnitude of the isometric force. The similar character of the two responses leads to uncertainty in designating the two motor axons. In ordinary insect skeletal muscles both would be considered 'fast' axons. The response of the extensor tibiae muscle of the locust to the slow axon, although many hundreds of times weaker than to the fast, nevertheless has the character of a small twitch, although it is extremely weak. The differences in response of the spiracular closer compared with the jumping muscle may be differences of degree, rather than of

kind, depending principally on the extent to which the axon giving the weaker contraction innervates the muscle fibres. For convenience the same notation will be adopted, the axon giving the larger contraction being termed the fast axon and the other the slow.

Electrical potentials

The resting potentials encountered have been rather variable, from 40 mV to about 70 mV, averaging just under 60 mV in a good preparation. This value is comparable to that obtained in ordinary skeletal muscle of locusts bathed in the same saline medium (HOYLE, 1955). There was no obvious difference between the small fibres of the core and the large ones of the outer region, although no systematic investigations have so far been made on the two kinds. Most observations were confined to the visualizable outer fibres.

The electrical excitability of the membranes has been investigated in preliminary experiments in the usual manner, with double-barrelled electrodes, one being used for recording and the other for passing current across the membrane. Rectangular pulses were applied using radio-frequency coupling units to isolate them. Anodal currents result in increased potential, there being a linear relationship between current and voltage up to membrane potentials of about 100 mV, after which there is a departure, indicating increasing membrane resistance. With cathodal currents there is a small decrease in the resistance of the membrane. In many fibres depolarization did not result in any significant departure from a time-course determined by the electrical properties of the membrane. Since it is unlikely that all such fibres are either damaged or exhausted, this must be their normal condition. One way of interpreting the observations is to suppose that the muscle fibres have few, or very small, patches of electrically excitable membrane (cf. CERF *et al.*, 1959). Other fibres developed a minute, heavily damped oscillation (Fig. 5), but the magnitude of these electrically excitable responses did not exceed 10–12 mV. These more electrically excitable fibres were encountered in the core, more often than in the outer layer. They are, however, very feebly responsive, even when compared with ordinary skeletal muscles of insects, where the electrogenic action (GRUNDFEST, 1957) is too feeble, even when maximal, to be regenerative and propagated. The muscle resembles the electroplax of *Raia clavata* in being essentially electrically inexcitable (GRUNDFEST, 1957).

Neuromuscular transmission

The fast axon—The electrically inexcitable nature of the muscle membrane is reflected in normal transmission. The responses to stimulation of the fast axon are, in most muscle fibres, only end-plate potentials (e.p.p's.). These vary in magnitude from 10–60 mV (Fig. 6). The larger ones may almost reach the zero level, and include a small component due to an abortive spike corresponding to the small electrically excitable response obtained with cathodal currents. This component may, in turn, lead to a small overshoot. These large e.p.p's. do not

facilitate, nor do they summate, and they may decline in magnitude during repetition. The smaller potentials summate in varying degrees and some of them show a small extent (up to $\times 2$) of facilitation. All the responses tend to fluctuate in height during repetition. In fibres capable of giving electrically excitable responses, the summated, and/or facilitated e.p.p's. may give rise to a tiny spike response (Fig. 8).

The height of the e.p.p. may be made to increase by raising the membrane potential, showing that as in other systems the e.p.p. is due to the development of a short-circuit across the membrane potential in the end-plate regions. A chemical transmitter is implicated, leading to a membrane reaction. The magnitude of this reaction differs from junction to junction (or fibre to fibre?), possibly as a result of different extents of release of the transmitter substance. Gradations of this kind show up very clearly in the fibres of this muscle, with their lack of secondary, active-membrane responses.

The slow axon—Responses to the slow axon are present, like those of the fast, probably in all the muscle fibres. If we compare this muscle with others in the locust which have been investigated (HOYLE, 1957), it becomes apparent that in doubly-innervated muscles the degree of double innervation (as indicated by the electrical responses) varies according to functional needs. The extremes are: every fibre innervated by two axons on the one hand, and each fibre innervated by only one axon on the other. In the tiny muscle of the spiracle, the slow axon excites all the muscle fibres. In the large, powerful jumping muscle only 30 per cent, and in the ordinary leg muscle about 50 per cent of the muscle fibres are doubly innervated, although in these muscles each fibre receives endings from the fast axon.

The slow potentials in the closer muscle fibres are always smaller than the corresponding fast ones, being from 40–80 per cent with the majority being about $\frac{2}{3}$ the height of the fast potentials in the same fibres. The times for decay to half are the same as those for the corresponding fast axon responses when the latter are only e.p.p's. Thus a small fast response closely resembles a large slow one (Fig. 7). The slow response could be due to the release of a transmitter substance identical with that of the fast transmitter, but released in smaller quantity. The neuromuscular junctions are just at the limit of resolution with the light microscope, so accurate work on them will require the use of the electron microscope. But it appears probable, from my investigations with the light microscope, that the final terminations consist of branches of the slow and fast axons lying side by side, sharing grooves in the surfaces of the muscle fibres. It is not, therefore, necessary to postulate differently reactive patches of postsynaptic membrane to explain differences in transmission by the slow and fast axons.

In studies of the homologous muscle in the cockroach CASE (1957) found that it could be excited to contract by electrical stimulation applied to the muscle directly, for up to three months following section of the nerve. In all examples of responses of insect muscle to so-called direct stimulation which have been analysed carefully it has transpired that the excitation is in fact indirect, via the fast axon.

VOL. 3 1959

Whether or not the nerve in the muscle actually degenerates following excision needs to be determined before it is possible to conclude that the cockroach muscle is electrically excitable.

Tension

The present recording method permits a degree of shortening which is small compared with the amount which the muscle shortens during closure of the valves. It can be used to give an approximate value for the isometric tension. Over a range of pen deflexion up to the extent where pen distortion starts there is a linear relationship between the tension and the peak deflexion. The amplification was adjusted to utilize this range in the experiments.

Values obtained for the tension developed in a twitch produced by stimulating the fast axon range from 0·05 to 0·14 g. On stimulating the slow axon the twitch tensions ranged from 0·02 to 0·05 g and were usually about one third of those obtained on stimulating the fast axon (Fig. 9a). This is in contrast to the relative magnitudes of the electrical effects, where the end-plate potential on stimulating the slow axon is usually about $\frac{2}{3}$ of that following stimulating of the fast axon.

It was found that when the stimulus pattern was changed from single shocks to pairs, with the two shocks closely-spaced, the resulting twitch tension was much greater. This was the case even when the shocks were spaced so close together (3–4 msec) that there was very little electrical summation. Furthermore, the enhanced mechanical response continued to increase in height during continued stimulation by pairs of shocks for as long as 20–30 sec, reaching 2–2½ times the twitch height to a single shock (Fig. 9b). These phenomena are probably to be interpreted in terms of the short duration of the active state in the muscle, and the taking up of internal 'slack' or series compliance (HILL, 1951).

The tetanus fusion frequency for the fast axon response is about 60/sec. It has not been possible to determine that for the slow.

Normal control mechanism

The control mechanism of the spiracle in the nearly intact animal has been investigated by making a similar preparation of the spiracular muscle but leaving the spiracular nerve intact and connected to the ganglion and also leaving the leg nerves unsevered. The head, abdomen, pro-, and metathoracic ganglia were then removed one by one, reflex responses being tested at each stage, and the spontaneous activity carefully investigated. The reflex responses of the spiracle to stimulation of various parts of the body are either opening or closing, i.e. the tension in the muscle may fall, partly or to zero, or it may rise. In these investigations only two stimuli have been found which cause reduction or arrest of discharge, i.e. opening of the spiracle; they are the gas carbon dioxide applied to the open spiracle, and tactile stimuli applied to the abdomen when these are strong enough to cause extensive abdominal contraction. The second response can be delayed by slowing down the contraction forcibly, with forceps. It appears to be caused by the excitation of proprioceptors in the abdomen at a position of extensive

VOL.
3
1959

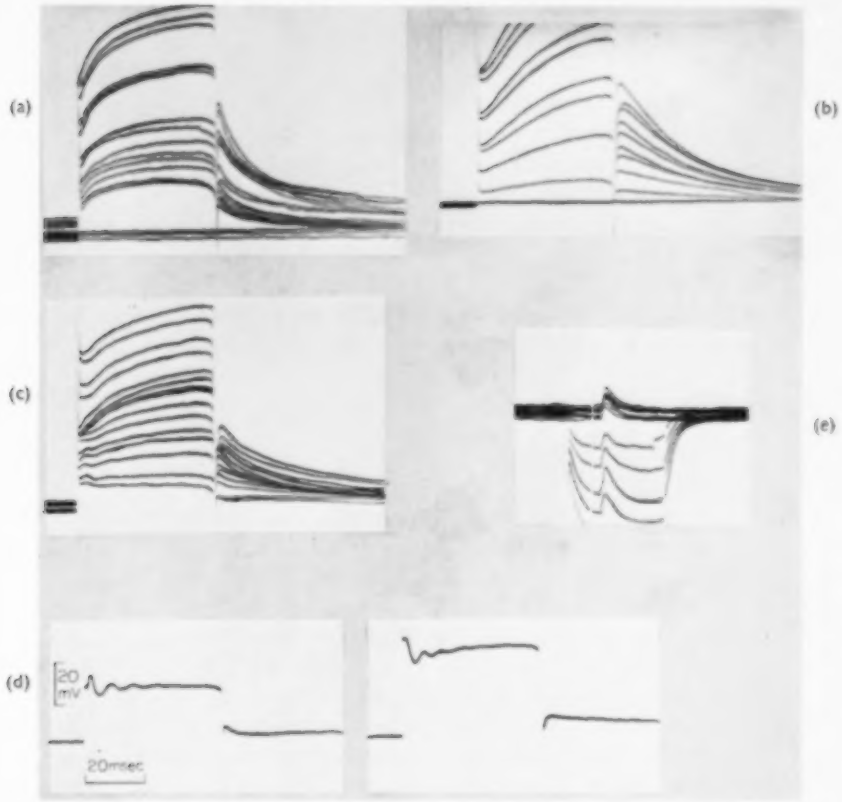


FIG. 5. Electrical responses of the muscle membrane to direct electrical stimulation. (a, b, c, d) Depolarizing pulses applied to 4 different muscle fibres. There is a small active response in (c), a very small one in (a) and hardly any at all in (b). In (a) there is a larger, abortive spike, followed by a heavily-damped oscillation. (e) Polarizing pulses applied during the elicitation of a response to the fast axon, which was a small e.p.p. The potential is enhanced following the raising of the membrane potential.

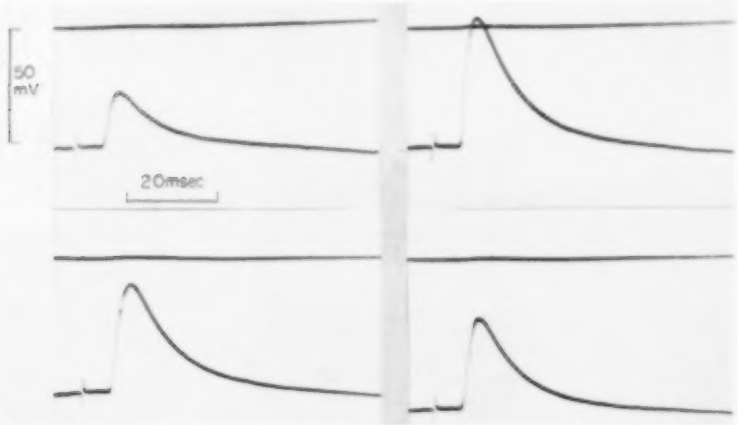


FIG. 6. Four fast responses recorded from different muscle fibres of the same muscle (19–20° C). The upper horizontal trace of each pair indicates zero membrane potential. The lower trace is the intracellular potential.

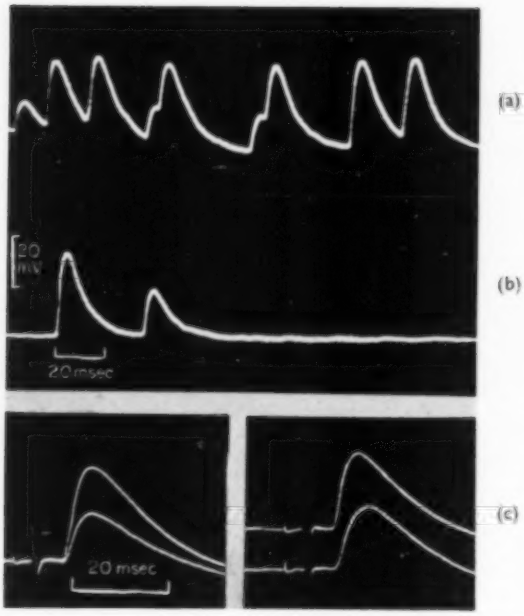


FIG. 7. Electrical responses to slow and fast axons (19–20° C). (a) and (b) Potentials occurring 'spontaneously' with the connexion to the ganglia intact. The larger potentials are fast responses, the smaller ones slow responses. The fast response was a large one and there is no summation of succeeding responses. (c) Two pairs of slow and fast responses obtained by separate electrical stimulation of the axons, each in the same muscle fibres. Those of the response shown on the right were separated for clarity. The larger (upper) response is the fast one in each case.

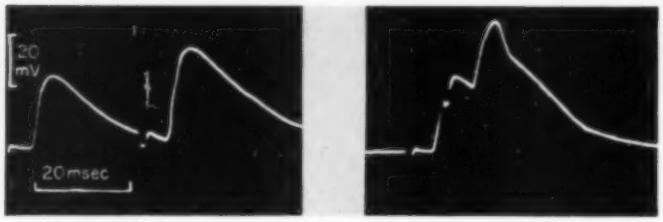


FIG. 8. Paired fast electrical responses. (a) The single response was a small one hence there is both facilitation and summation of the second response. (b) The same as (a) but with closer spacing. Note the inflexion in the rising phase and the abortive spike.

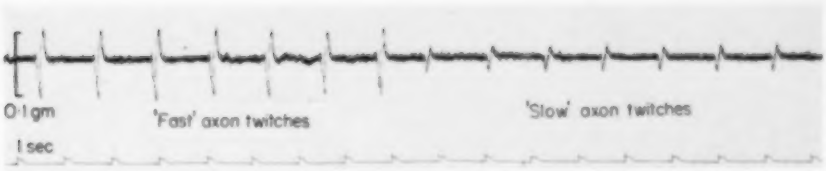


Fig. 9 (a)

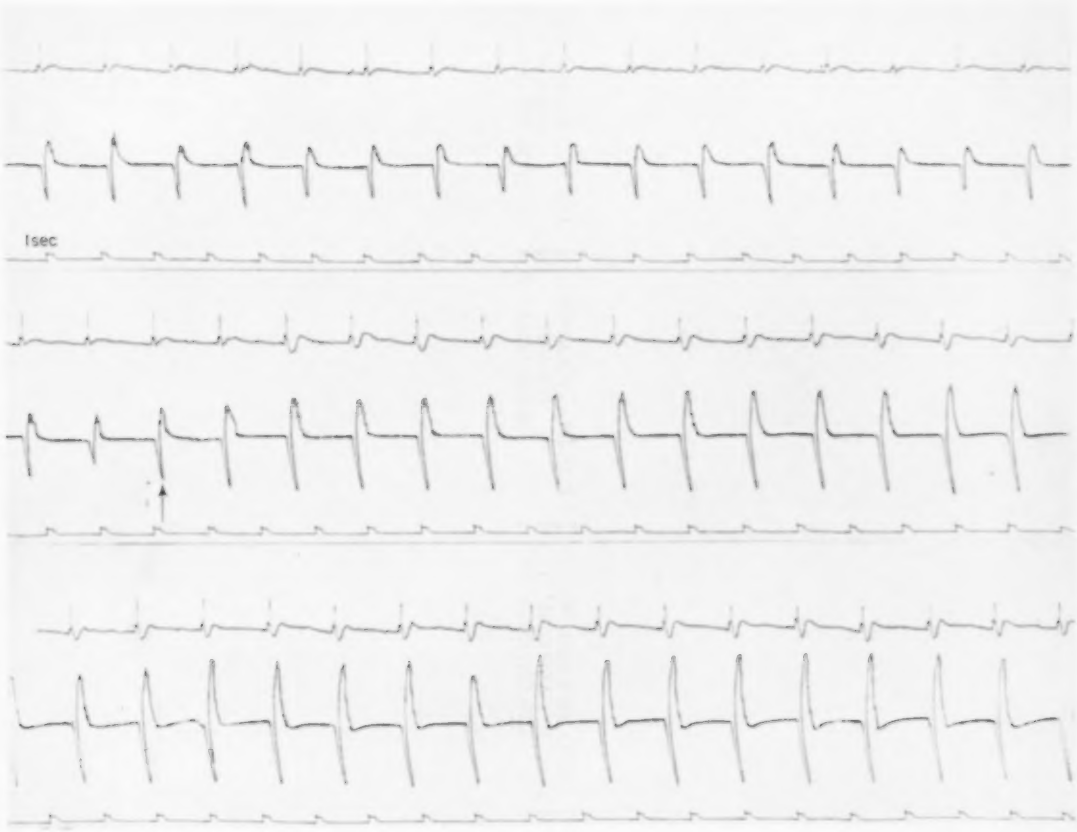


Fig. 9 (b)

FIG. 9. Mechanical responses recorded from the apodeme of the closing muscle (19–20° C). (a) Fast and slow twitch responses to stimulation of the two axons innervating the closing muscle. The first seven responses are to the fast axon alone and the rest to the slow. (b) Effect of close-pairing of stimuli on the twitch response. Upper trace—electrical potentials from single muscle fibre. Lower trace—tension record of whole muscle. Time in sec. Single shocks were applied from start up to the arrow when the pattern of stimulation was changed abruptly to close pairs, delivered at the same rate.

VOL.
3
1959

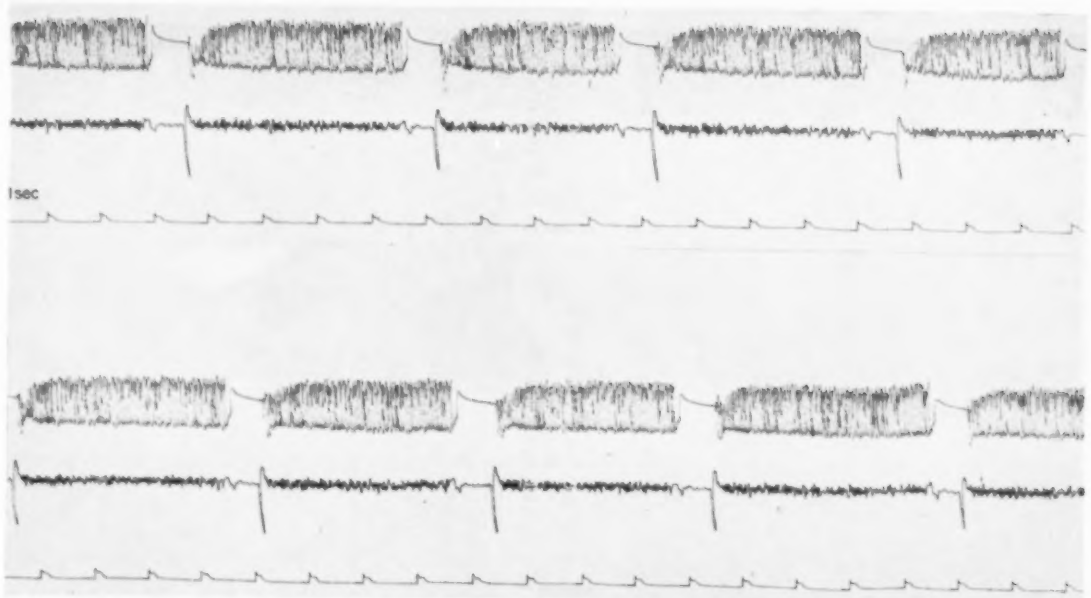


FIG. 10. Spontaneous activity in preparation with spiracular and median nerve intact. The connexions of the mesothoracic ganglion with the rest of the nervous system had been severed. Upper trace—electrical potentials from a single muscle fibre. Lower trace—tension record of whole muscle. Time in seconds. Note the oscillations in tension occurring during the partial tetanus which causes closing. The activity of the closing muscle is abruptly interrupted at intervals to allow regular periodic opening of the spiracle.

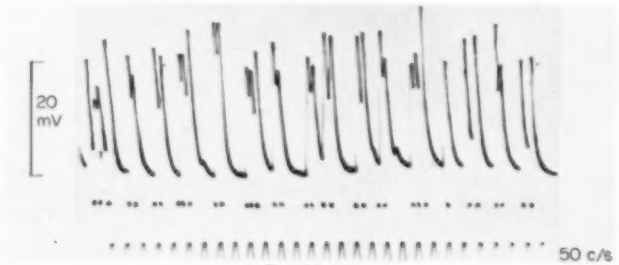


Fig. 11 (a)

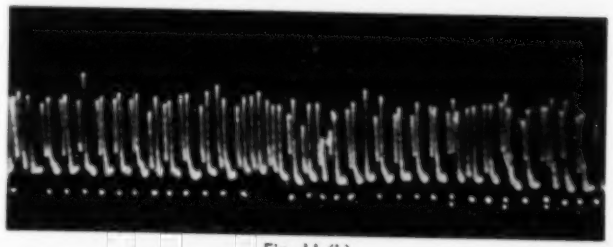


Fig. 11 (b)

FIG. 11. Nature of the impulse discharge in periods of closing during the normal rhythm. End-plate potentials recorded from a single muscle fibre. The majority are fast-fibre potentials. Note the high proportion of close pairs of potentials. In (a) each potential is indicated by a single dot (.). In (b), which was on a slower time-base, close pairs are indicated by a single dot (.), triplets of potentials by a double dot (:), complete record in (b) = 1 second (.).

VOL.
3
1959

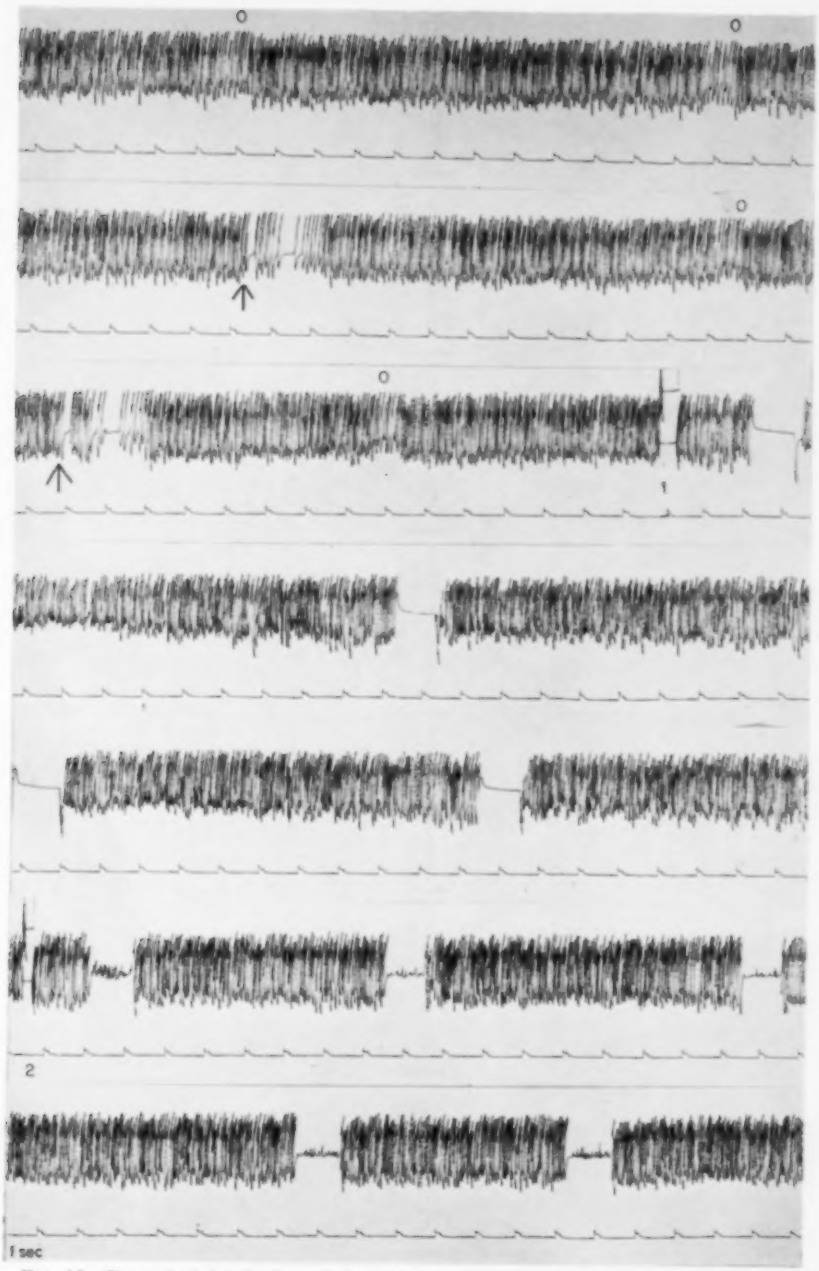


FIG. 12. Control of the rhythm of the spiracles. The record shown is a continuous one, except that the paper drive was stopped at 1 and 2 to allow time for further section of nerves, and subsequent settling down of the preparation. The record is of the electrical activity of a single muscle fibre of the spiracular closer with nerve connexions to the ganglion intact and follows the course of nervous discharge. The periods marked O coincide with opening of the spiracle (here it was only partial opening owing to a discharge of the slow axon). At the arrows the abdomen was touched. This caused inhibition of the discharge and opening of the spiracle. At the point marked 1, the abdomen was severed from the thorax. Note that a new, slightly faster rhythm results. The activity of the slow axon has stopped, and opening is complete. At 2, the connectives between the meso- and metathoracic ganglia were severed. The intrinsic rhythm of the controlling centre in the mesothoracic ganglion itself is now established. It was faster than that of either the metathorax or the abdomen in this preparation.

contraction. The action of carbon dioxide will be dealt with in a subsequent paper. All other effective stimuli, such as tactile stimulation of the hairs on the spiracular lips, cause the spiracle to close.

A dominant role in determining the spiracular rhythm is played by the abdomen. The spiracle remains closed during contraction of the abdomen in the relatively slow expiratory contraction movement. The closing continues during the initial phase of inspiration, until the air sacs in the thorax are slightly collapsed. At this stage the valves open briefly. During the later stages of inspiration they are closed. All this is part of the mechanism whereby air is caused to stream through the insect unidirectionally, entering the thoracic spiracles and leaving via the abdominal ones. If the movements of the abdomen are restricted or forced to slow down, then the spiracle follows the abdomen, continuing to open at the same point in the inspiratory extension of the abdomen. Thus the opening seems to be initiated by the movement of the abdomen.

If, during any part of the respiratory cycle, the abdomen is subjected to strong tactile stimulation, the valves open, allowing air, which comes under pressure in the insect during the resulting contraction of the abdomen, to escape from the thorax.

VOL. 3 1959

Before the 'preparation' has settled down, or if it is disturbed, the spiracles may remain closed or very nearly closed for long periods, despite continued vigorous respiratory movements of the abdomen. Analysis of the discharges shows that a closing of the valves may be due to either the fast or the slow axons firing alone, or to the two in combination. The frequency of discharge of the 'fast' axon is, however, virtually unaffected by tactile stimulation of the outside of the animal. Strong stimulation of the abdomen, which causes its cessation or reduction, is the only exception. It is the discharge of the slow axon which is always affected by the agencies which cause reflex spiracular closing. Stimulation of the hairs in the region of the spiracle, for example, results in a reflex discharge in the slow axon only.

The contraction of the closer muscle settles down to a regular rhythm in the intact-nervous-system preparation, with the duration of the open phase, like that of the closing, remaining constant for long periods (Fig. 10). Both opening and closing are usually abrupt, but if the slow axon continues to be active during the 'open' period, opening may be slower or even fail to occur. The durations of the two phases vary greatly from preparation to preparation. Closing may be for as short as 2·5 sec or as long as 25 sec. Opening may be as short as 0·3 sec or as long as 3 sec. Some examples are given in Table 1. Several different combinations of intervals have been observed. It will be seen from Table 1 that there is no correlation between the durations of the two phases, long closing sometimes being combined with short opening, or vice versa. Nor is this inverse relationship (which would be readily explicable on the basis of 'retention' or 'release' of carbon dioxide as the respective modes) constant either, since some short closings are associated with short openings, and the converse again applies. Further work is required on this point, in the whole animal. It looks as though there may be significant patterns into which the spiracular sequence may fall, possibly in relation

to immediate metabolic requirements which are in turn related to growth phases, e.g. egg production, digestion, etc. Broadly, there are three modes: quick, normal, and slow. In the former, ventilation is rapid, the spiracles are closed for only short intervals and remain open for relatively long ones. In the latter extreme, they are closed for long periods and only open for very brief ones.

TABLE 1. SOME VALUES FOR THE DURATIONS OF OPENING AND CLOSING OF THE SPIRACLE WERE MAINTAINED FOR SEVERAL MINUTES. VARIOUS PREPARATIONS, INTACT, AND WITH DIFFERENT EXTENTS OF ISOLATION FROM THE REST OF THE NERVOUS SYSTEM

Preparation	Duration of closing (sec)	Duration of opening (sec)
Thorax and abdomen	11	1·2
	4·6	1·7
	25	1·0
Thorax only	5	1·5
	9	2·2
	10	1·8
	19	0·5
	2·5	1·3
Mesothoracic only	7	1·5
	4	0·8
	6	1·6
	7·6	3·0

The detailed pattern of the nervous discharge, even when consisting solely of fast impulses, is always very irregular, but even a casual inspection shows that it is not a random one, such as would be given by a co-incidence counter operated by amplified thermal noise. Instead, a high proportion of close pairs of impulses is observed in all records (Fig. 11). As many as 90 per cent of the total number of impulses in a 6 sec burst may be delivered with an interval of only 4–9 msec, compared with an interval of 18 msec if they had been evenly spaced. In CASE's published work on the cockroach there are, in one record, eight close pairs (16 impulses) in a total of 32, i.e. half the impulses were delivered as close pairs, although CASE did not draw attention to this. WIERSMA and ADAMS (1950) have shown that the tension in some crustacean muscles is greatly increased if excitation is delivered via the motor nerve in close pairs, compared with the same number equally spaced. They remark that spacing might be used by the animal to vary tension development, but no evidence has hitherto been forthcoming that close pairs occur in the discharges of the appropriate motor nerves in the intact animal. The present observations constitute evidence that close pairing of impulses is in fact utilized by a nervous control mechanism.

The total number of impulses per second may reach 60 or 70 but usually falls in the region of 25–30. It occasionally falls to as low as 15/sec. But as with the other variables, durations of the discharge and of the silent period, the average

frequency tends to remain constant over quite long periods. The upper limits of the frequency of natural excitation of the muscle just reach the level of smooth tetanus, but the average value is well below the level required for complete fusion, and the oscillations in tension, especially those associated with pairs and triplets of impulses, are evident as a series of sharp peaks in the electromechanogram.

By contrast with the fast axon, the slow axon discharges in irregular, brief bursts, without the close spacing. Bursts occur when the preparation wriggles, or sometimes without obvious stimulation. Impulses may continue to appear right through the silent phase of the discharge of the fast axon, causing either continued closing or restricting the opening of the valves. Summation of fast and slow electrical responses may be appreciable, even showing complete addition, or it may be negligible. The magnitude of the summed electrical response is determined by the size of the fast response. If this almost reaches the zero potential or slightly overshoots no greater level of depolarization is reached by combining it with a slow one or with another fast response. If it is appreciably smaller than the resting potential then it sums with the slow. This supports the notion that the two axons liberate a common kind of transmitter substance into a shared subsynaptic region. When the reaction there is maximal, i.e. to a large quantity of transmitter substance released by the initial fast impulse, no further reaction (to a second fast impulse, or to a slow impulse arriving at the same time) can occur.

VOL. 3 1959

Local reflex network—In all preparations most of the electrical and all the mechanical activity of the spiracular apparatus ceased immediately the spiracular nerve was cut. Some miniature electrical potentials were seen, not associated with any mechanical activity. They might be due to spontaneous liberation of transmitter substance in 'quantal' units and be analogous to the spontaneous miniature e.p.p's. of vertebrate muscle (FATT and KATZ, 1952). But the present recording conditions, with a limited volume of fluid actually surrounding the muscle, permit a small pick-up by the intracellular electrode of potentials developed in adjacent muscle.

No evidence has been found that there might be a local nervous system, such as was postulated by BECKEL and SCHNEIDERMAN and others.

Closing movements—Mechanograms have been taken both from the apodeme, in the manner described earlier, and externally, from the valves themselves. The movements of the *Hyalophora* pupal valves were, under certain conditions, described as showing a 'fluttering' action. This is also characteristic of the locust valves when the frequency of discharge of the motor nerve is below that required for smooth tetanus. It is the tension peaks caused by the paired or triplet excitations, however, which are principally responsible for the fluttering activity.

DISCUSSION

The spiracular closer muscle is evidently unique in many ways, and may be profitably developed as a medium through which to investigate both specific insect muscle problems, and others of a more general nature. The discussion will be

confined to attempting to understand how the normal control sequence of the spiracular muscle may be achieved, in the light of the information available at present.

Following section of the connectives between the abdomen and the metathorax there is almost immediately a change in the rhythm of opening and closing. If the previous rhythm, determined by the abdominal movements, was a quick one (short periods of closing) then it is now slower. If the previous rhythm was a slow one then it is now faster (Fig. 12). The new rhythm is determined by the metathoracic ganglion. It is virtually unaffected, except momentarily, by severing the connexions with the prothoracic ganglion. But there is a further change in rhythm immediately following section of the connectives between the second and third ganglia. The rhythm is now determined by the mesothoracic ganglion itself. It has been slightly faster than that of the metathoracic ganglion in most of the present experiments. Thus there are at least three centres which are capable of rhythmic activity, showing a pattern within the range encountered in normal respiratory movements. They have a hierarchical connexion in the direction: abdomen dominates metathorax dominates mesothorax.

In his account of the co-ordination of the rhythmical respiratory centres in the same species, FRAENKEL (1932) concluded that the activity of the pacemaker which happens to be fastest at the time dominates the rest. This opinion has not been substantiated by the present findings. The opening rhythm in the preparation having its nerve connexions with the abdomen intact has been as slow as one opening in 25 sec. But no value in excess of 10 sec has been obtained after isolation of the mesothoracic ganglion from other nervous connexions. There is thus no doubt that either there is no intrinsic rhythm in the mesothoracic ganglion in the intact animal, or else that one is present, but it is suppressed by the more posterior centres.

If we accept the latter view, we must realize that the situation is unusual, in that the rhythm is not dominated by the centre having the fastest rate, as in the control of the vertebrate heart for example. The pacemaker in each ganglion must be affected by that of the one immediately posterior to it. As long as the latter is discharging motor impulses the action of the anterior pacemaker is suspended, to follow the posterior one. This does not mean that the anterior ganglia cannot affect the posterior ones, speeding them up in response to a thoracic deficiency of oxygen, for example. But such an effect would be a direct one on the dominant posterior pacemaker, brought about by nerves passing directly backwards, and not indirectly, by first acting on the local pacemaker. This would only serve to disrupt the co-ordination of spiracular opening and interfere with the movement of air through the body.

The abruptness with which the motor discharge starts and stops suggests that the pacemaker may be an inhibitory cell acting on an otherwise continuously active motor cell, the periods of maximum inhibition corresponding to cessation of the motor discharge and opening of the valves. The inhibitor would in turn be inhibited by the discharge of the fast nerve of the more posterior spiracular control centre.

The rhythm of the mesothoracic ganglion, actuating the fast axon supplying the closers of the second pair of spiracles, is not affected either by forcing the valves to remain closed during an opening phase, or by forcing them open from the closed position. It is thus not dependent on sensory feed-back from the valves themselves. All the paired nerves emanating from the ganglion can be cut one by one without altering it. The most conspicuous nervous influence on the fast motoneurone (F) is thus that coming from the metathoracic ganglion, which is in turn affected by the abdomen. The control sequence is illustrated schematically by a simple neurone model in Fig. 13.

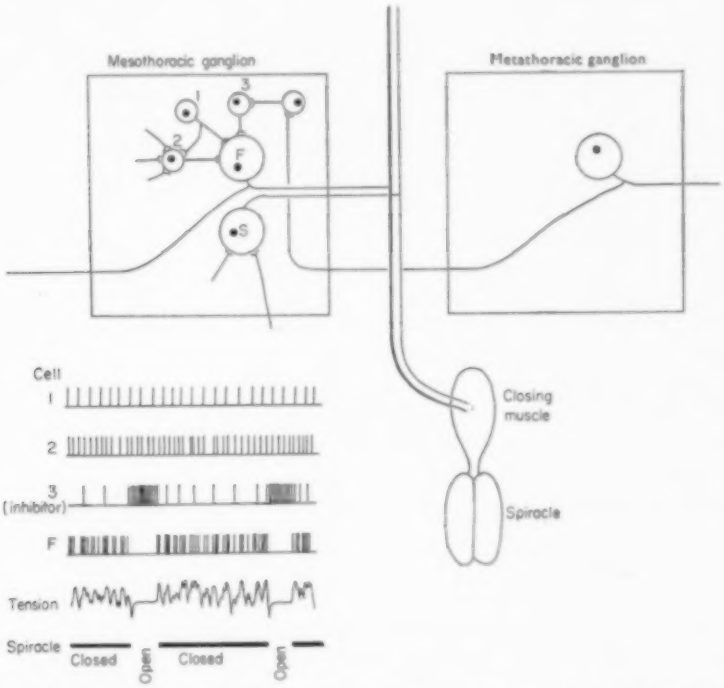


FIG. 13. Hypothetical scheme for control mechanism of spiracle. (For explanation see text.) The two nerve cells, slow and fast, are in reality present. The other cells represent a simple model which might account for the patterns of discharge actually found in the fast axon. The pattern of their discharges is illustrated schematically at the lower left.

The role of the slow axon is by no means clear. One advantage of using electrical recording from the muscle fibres is that discharges of both axons are readily distinguishable, except when the frequency of discharge is very high. Recording from the nerve can be misleading unless the action potentials are of different heights and can be accurately designated as belonging to the slow or fast axons. Sometimes the potentials recorded in the muscle are entirely fast ones, and the operations just described can be made without bringing in the slow axon,

VOL. 3 1959

although mechanical disturbance of the valves (forced opening) tends to cause S to fire. But the S neurone quite often does discharge during the normal rhythm of opening and closing, with silent periods coinciding with opening. The firing of the S axon with the normal rhythm could be due to central nervous interplay in the rhythmic centre, but it is thought more probable that it is the result of reflex excitation caused by the closing movements.

The durations of opening and closing vary greatly from preparation to preparation. They are thus set by metabolic influences within the pacemaker itself. Continuous spontaneous nervous activity is evident in isolated nerve cords of insects for some days (ROEDER, 1948), but many influences could be partly responsible for initiating such activity and the evidence does not seem conclusive that there are spontaneously active motor cells. But there seems to be a very good case, in the present instance, for regarding the activity of the pacemaker as truly intrinsic, arising *de novo* in a manner comparable to that found in some sense organs.

The intervals between succeeding impulses in the single fast motor nerve cell (referred to subsequently as the F cell) are so variable that it seems unlikely that it is itself a spontaneously active cell, since most such cells tend to fire with rather regular intervals between impulses. We will suppose, instead, that it is little more than a relay, transmitting excitatory patterns received from other cells. The only regular feature of its discharge is the large proportion of close pairs of impulses, and this has to be explained. The timing of opening and closing can be interpreted as determined by an inhibitory cell (I) which discharges (in a commonly observed pattern) with regularly fluctuating frequency. When the frequency exceeds a threshold value, F is inhibited completely, corresponding to opening. The cell (I) is in turn driven by the metathoracic ganglion.

Two more cells are required to complete the picture. One of them (1) is a simple, spontaneously discharging cell exciting F. It is hardly affected by external influences. It also synapses with a relay-type cell (2) which in turn excites F. This inter-neurone can account for the close pairs of impulses. Cell 2 also receives excitation from other cells in the ganglion, some of them firing rather randomly. These impulses, in so far as they pass through 2, combine with those from 1 to excite F and determine the discharge pattern of closure.

The slow motor nerve cell (S) is shown as not being connected to the circuits affecting F. It is involved in various local reflexes and can cause partial or complete closure of the spiracle independently of the discharge in the F cell. A branch of F is shown going forwards. A branch of this kind from some part of the control centre in the mesothorax must be postulated, since the discharge of the comparable centre in the prothoracic ganglion appears to be driven by that of the mesothorax, just as the latter is in turn driven by the metathorax. In the model (Fig. 13) the activity of the final pacemaker, the inhibitory cell (I), must be itself inhibited as long as the metathoracic F cell is firing. This might be achieved by interpolating an inhibitory internuncial neurone between the metathoracic centre and the mesothoracic (1) cell. F becomes active as soon as the inhibitory discharge of (1) falls below the threshold frequency. Thus the mesothoracic spiracle cannot open,

except under the influence of a high local concentration of carbon dioxide, when the other spiracles are closed. (1) becomes active as soon as the more posterior spiracle opens.

When the pacemaker cell (1) is freed from the inhibitory influence of the abdomen and metathorax it discharges spontaneously, with a regularly-fluctuating rise and fall of frequency, an oscillation which may depend on negative feed-back within the ganglionic neurone chains. Thus the cell F is caused to be inhibited at regular intervals and the mesothoracic ganglion assumes an autonomous rhythm of control of the opening and closing movements of its own spiracles. The presence of the potentially autonomous control centre in the mesothorax may be a relic of the time when a simple condition of independently acting segments prevailed in the class. The retention of the controlling network may be fortuitous, but it does confer a measure of independence still, particularly in regard to closing, which is regulated by local as well as by total requirements. The spiracle also reacts in a truly local manner to the presence of a high concentration of carbon dioxide, as will be shown in a subsequent paper (HOYLE, 1960). Indeed, the action of the gas completely over-rides the influence of both the slow and fast excitatory axons, so that the spiracle cannot remain closed in the face of a high carbon dioxide concentration, no matter how great the motor excitation.

VOL. 3 1959

SUMMARY

(1) The anatomy and innervation of the closing muscle of the second spiracle of the locust, *Schistocerca gregaria*, are briefly described. From the muscle and its associated spiracle, a preparation can be made which permits the physiological functioning and nervous control mechanisms of the spiracular apparatus to be studied in detail.

(2) The neuromuscular mechanisms were examined with the aid of electrical stimulation of the motor nerve and intracellular recording from the closer muscle. The closer muscle receives two motor axons, each of which appears to give responses in all the muscle fibres.

(3) Each of the axons promotes a twitch contraction when stimulated with a single shock. One of the responses is only one-third the magnitude of the other; it is referred to as the slow response, the other being called the fast response. The corresponding axons are called fast and slow by analogy with the terms used in doubly-innervated crustacean muscle, although the differences are here thought to be due to differences in force, rather than speed, of contraction.

(4) The electrical responses consist largely of end-plate potentials. There are secondary responses in a few fibres but they are abortive and do not exceed 12 mV. Electrical responses occurring to stimulation of the slow axon are similar in time-course to those of the fast, but they are about two-thirds their magnitude, in most fibres.

(5) The normal control mechanism of the spiracular muscle was investigated, starting with the nearly-intact animal. The rhythmic opening and closing movements are determined by periods of firing or rest in the fast axon. These are

controlled in turn by the abdomen, the metathoracic ganglion, and the mesothoracic ganglion itself, in that order of influence.

(6) The closing nervous discharge is an irregular one, but with a high proportion of close pairs of impulses. Its average frequency in different preparations varied from 15 to 80/sec. The durations of opening and closing remain constant over long periods, but they vary a great deal from preparation to preparation.

(7) A neural model which could account for some of the observed features of the discharge is proposed and discussed.

(8) No evidence was found for a peripherally-located sensory-motor nervous system affecting the functioning of the spiracular muscle.

REFERENCES

BECKEL W. E. and SCHNEIDERMAN H. A. (1957) Insect spiracle as an independent effector. *Science* **126**, 352–353.

BUCK J. (1958) Cyclic CO_2 release in insects. IV. A theory of mechanism. *Biol. Bull., Woods Hole* **114**, 118–140.

CASE J. F. (1957) The median nerves and cockroach spiracular function. *J. Ins. Physiol.* **1**, 85–94.

CERF J., GRUNDFEST H., HOYLE G., and MCCANN F. V. (1957 and 1959) The nature of electrical responses of doubly-innervated insect muscle fibres. *Biol. Bull., Woods Hole* **113**, 337–338, and in press.

FATT P. and KATZ B. (1952) Spontaneous subthreshold activity at motor nerve endings. *J. Physiol.* **117**, 109–128.

FRAENKEL G. (1932) Untersunchungen über die koordination von Reflexen und automatisch-nervösen Rhythmen bei Insekten. IV. Über die nervosen Zentren der Atmung die Koordination ihrer Tätigkeit. *Z. Verg. Physiol.* **16**, 444–462.

GRUNDFEST H. (1957) The mechanism of discharge of the electric organ in relation to general and comparative electrophysiology. *Progr. Biophys.* **7**, 1–85.

HILL A. V. (1951) The effect of series compliance on the tension developed in a muscle twitch. *Proc. roy. Soc.* (B) **138**, 325–338.

HOYLE G. (1953) Potassium ions and insect nerve muscle. *J. exp. Biol.* **30**, 121–135.

HOYLE G. (1955) Neuromuscular mechanisms of a locust skeletal muscle. *Proc. roy. Soc.* (B) **143**, 343–367.

HOYLE G. (1957) The nervous control of insect muscle. In *Recent Advances in Invertebrate Physiology* (Edited by SCHEER). University of Oregon Press.

HOYLE G. (1960). The action of carbon dioxide on an insect spiracular muscle. *J. Ins. Physiol.* **4**. In press.

KUFFLER S. W. and VAUGHAN WILLIAMS E. M. (1953) Small-nerve junction potentials. The distribution of small motor nerves to frog skeletal muscle, and the membrane characteristics of the fibres they innervate. *J. Physiol.* **121**, 289–317.

MILLER P. (1959) Ph.D. thesis, University of Cambridge. In preparation.

PUNT A. (1950) The respiration of insects. *Physiol. comp.* **2**, 59–74.

ROEDER K. D. (1948) The effect of potassium and calcium on the nervous system of the cockroach, *Periplaneta americana*. *J. cell. comp. Physiol.* **31**, 327–338.

SCHNEIDERMAN H. A. (1956) Spiracular control of discontinuous respiration in insects. *Nature, Lond.* **177**, 1169–1171.

WIERSMA C. A. G. and ADAMS R. T. (1950) The influence of nerve impulse sequence on contractions of different crustacean muscles. *Physiol. comp.* **2**, 20–33.

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 395 *to* 401. *Pergamon Press Ltd., London. Printed in Great Britain*

THE REACTION OF THE COCKROACH *PERIPLANETA AMERICANA* L. TO BODILY STRESS AND DDT

J. P. HESLOP and J. W. RAY

Pest Infestation Laboratory, Slough, Bucks.

(*Received* 6 *April* 1959)

Abstract—The effects of DDT and bodily stress upon the oxygen consumption and neuromuscular system of *Periplaneta americana* L. have been investigated and compared. Tolerance of DDT in two strains of cockroaches has been shown to be correlated with tolerance of stress. A tentative hypothesis as to the course of the insect's reaction to poisoning and to stress is put forward and briefly discussed.

VOL.
3
1959

INTRODUCTION

WHEN an insect is treated with a poison such as DDT it is subjected to a period of bodily stress. In the interpretation of the biochemical and physiological disturbances associated with the action of the insecticide it is important to know the effects of stress alone. This may enable the specific effect of the insecticide to be distinguished from the non-specific consequences of poisoning. BEAMENT (1958) recently described a condition of paralysis in the cockroach caused by prolonged maltreatment of various kinds. He showed the presence of a toxic substance in the haemolymph of the paralysed cockroach which would induce paralysis when injected into a normal one. The experiments described in this paper were designed to throw more light on the phenomenon of paralysis and to show its relation to the condition of prostration produced by DDT poisoning. Throughout this paper the term 'paralysis' has been used to describe collapse following physical stress and 'prostration' to describe collapse due to DDT poisoning.

MATERIALS AND METHODS

Adult male *Periplaneta americana* were used in all experiments. They were handled without the use of anaesthetics but the minimum chill coma was used before DDT application or tying down. The insects were never completely immobilized. Except where stated, the cockroaches were reared at the laboratory on a diet of rolled oats, toppings (a wheat bran fraction), fishmeal and dried yeast, 45:45:5:5 by volume. They received food and water *ad lib.* up to the time of experiment. Through the kindness of Dr. J. W. L. BEAMENT, cockroaches for one series of experiments were obtained from the Zoology Department, University of Cambridge.

Continuous records of oxygen consumption were made on individual animals at 27°C and 50 per cent relative humidity in two electrolytic recording respirometers kindly loaned by Mr. F. P. W. WINTERINGHAM. A brief description of the

technique has been published by Winteringham (1955) and a detailed account is being prepared for publication (personal communication).

The cathode ray oscilloscope apparatus used to observe electrical impulses in the nerve cord was of conventional design. Two platinum hook electrodes placed beneath the cord between the second and fourth abdominal ganglia were used to pick up the impulses. Physical stress was imposed (cf. Beament, 1958) by immobilizing the animals on their backs, or with a powder mill made by tying 4-oz powder jars to the spokes of a wheel 14 cm from the centre and rotating it at 20 rev/min. In the respiration experiments individual cockroaches were tied to drilled Perspex sheets with cotton thread. One thread passed across the thorax and each leg was separately secured by a thread tied between femur and tibia.

The Ringer solution used was prepared as described by Hoyle (1935).

RESULTS

The terms used by various workers to describe the same stages of DDT poisoning sometimes differ. Those used in this paper are defined in Table 1. Cockroaches paralysed by physical stress show outward symptoms indistinguishable from prostration and late prostration but do not exhibit earlier symptoms.

Table 1. Terms used to describe the stages of DDT poisoning

Descriptive term	Definition—stage of poisoning
Initial tremors	Slight ataxia, 'hangover' effect—jumps when jar is tapped
Tremors	Still able to stand but limbs tremble and twitch
On back kicking	Unable to stand, violent convulsions
Prostrate	Lies on back, legs twitch
Late prostrate	Very desiccated, shortened abdomen, darkening of cuticle, curling of antennae and caudal cerci, few very slight movements

Respiration experiments

Comparative measurements of oxygen consumption have been made on cockroaches treated topically with 100 μg DDT in 5 μl acetone and on animals tied on their backs to Perspex sheets. Out of 14 tied-down cockroaches examined in the respirometer 4 became paralysed. Fig. 1 (a) is a graph of oxygen consumption against time for two tied down cockroaches one of which became paralysed. In Fig. 1 (b) is shown the oxygen consumption of one DDT-treated and one control insect. The cockroaches which were tied down but did not become paralysed showed a similar respiratory pattern to the controls which were free to move at will within the respirometer. Even when they struggled violently against their bonds they did not respire nearly so fast as the DDT-treated ones or the stressed animals which became paralysed. The surge of respiration preceding prostration or paralysis followed a similar pattern in all cases but the time interval between treatment

and the burst of respiration was less constant in the paralysed animals. No intermediate respiratory pattern between the normal and that preceding paralysis or prostration has been found. The onset of paralysis seems therefore to be an all or none phenomenon.

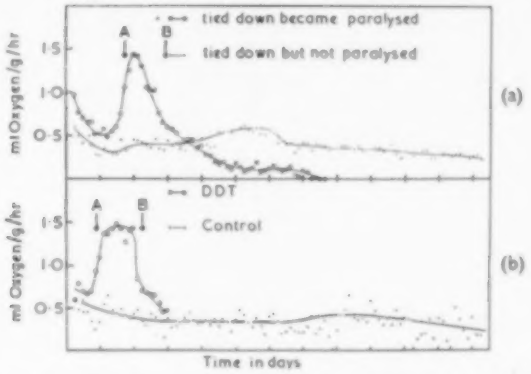


FIG. 1. Graphs of oxygen consumption against time for individual cockroaches.

TABLE 2. OXYGEN CONSUMPTIONS OF INDIVIDUAL COCKROACHES (*a*) TIED DOWN, (*b*) TREATED TOPICALLY WITH 100 μg DDT IN ACETONE OR UNTREATED. POINT 'P' IS THE TIME OF PROSTRATION OR PARALYSIS

(*a*)

Paralysed		Not paralysed	
Days to 'P'	O_2 to 'P' cm^3/g	Time (days)	O_2 used cm^3/g
3½	67·5	13	107·5
5	102·8	13	109·8
2½	55·6	13	109·9
½	20.2		

(*b*)

DDT		Control	
Days to 'P'	O_2 to 'P' cm^3/g	Time (days)	O_2 used cm^3/g
2½	55·5	13	106·6
2½	64·3	17	131·9
2½	69·4		
2½	60·0		

Table 2 gives the total oxygen consumptions of cockroaches up to the point of paralysis or prostration and of controls kept in the respirometers without food or water for two weeks. Owing to the difficulty of deciding the exact time of collapse

of insects tied in an unnatural position in a respirometer, in the case of tied-down cockroaches the point of paralysis has been taken as the time at which their respiratory rates fell below those of the controls. Five cockroaches which had remained in the respirometers for thirteen days or more without food or water showed no signs of paralysis (Table 2). They had all consumed more than 106 ml oxygen per gramme body weight. It is therefore unlikely that the collapse of prostate or paralysed cockroaches which frequently occurs when much less oxygen has been used is due to complete exhaustion of endogenous reserves. The insects may become unable to utilize their remaining reserves due to a failure of transport mechanisms or a biochemical lesion.

Tolerance of stress

Fifty Cambridge and 31 P.I.L. cockroaches were each dosed with 20 μg DDT in 20 μl acetone applied topically to the ventral abdomen and were placed in separate 1 lb jam jars with food and water at 25°C. All showed initial tremors within an hour and marked tremors 15 hr after treatment. As shown in Fig. 2 the Cambridge stock were much quicker to become prostrate.

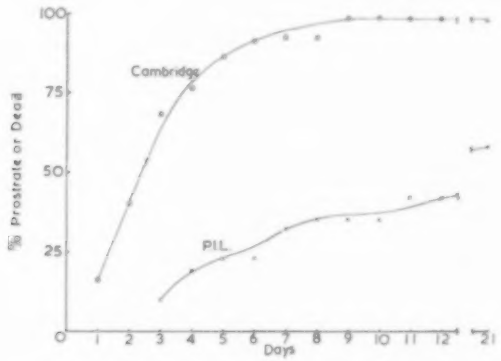


Fig. 2. The effect of DDT on two strains of *P. americana*. 20 μg DDT was applied topically in acetone to each individual at zero time.

A number of animals from each strain were subjected to stress in a powder mill. The results are shown in Table 3.

Table 3. Effect of powder mill stress on *P. americana*

	Cambridge strain	P.I.L. strain
Time of exposure	8 or 12 hr	18½ hr
No. animals used	12	18
No. paralysed after 8 days	12	0
No. paralysed after 19 days	12	1

The P.I.L. strain is more difficult to paralyse by means of a powder mill than the Cambridge one. It is also less susceptible to DDT. The difference in susceptibility to these two forms of stress may be an indication of vigour tolerance as defined by HOSKINS and GORDON (1956). The P.I.L. strain may be able to withstand some of the secondary effects of DDT poisoning although there is no indication that they are resistant to the primary action of DDT.

Neuromuscular disturbances

The impulses passing along the ventral abdominal nerve cord of a number of cockroaches have been examined. In those treated with DDT, trains of impulses are always observed at the stage of tremors and frequently continue after prostration. Another feature of DDT poisoning is facilitation in ganglia. This is readily demonstrated in *P. americana* using the reflex arc from the hair receptors on the caudal cerci via synapses in the sixth abdominal ganglion to the thoracic ganglia. The impulses are carried up the cord by giant fibres and are therefore easily monitored. When facilitation is present the ganglion continues to discharge for up to a minute after a single short air puff stimulus to the receptors. Table 4 contains our observations on the occurrence of trains of impulses in the nerve cord. Facilitation was clearly seen in two paralysed and five prostrate cockroaches. It can be obscured by hyperactivity and trains of impulses in the cord.

VOL. 3 1959

TABLE 4. THE OCCURRENCE OF TRAINS OF IMPULSES IN THE VENTRAL ABDOMINAL NERVE CORD OF *P. americana*

Condition of animal	No. examined	No. showing trains
Prostrate (DDT)	45	30
Paralysed (stress)	13	8
Normal	90	0

Tremors of the limbs were found at the early stage of paralysis in all of 83 cockroaches. The later stages of paralysis were indistinguishable from late prostration.

The authors independently observed that the respective trains of impulses in cords of paralysed and prostrate cockroaches differed qualitatively. Those of paralysed insects appeared to be less regular and less persistent. Moreover, after thorough washing of the body cavities of paralysed cockroaches with Ringer solution, in several cases trains entirely ceased and facilitation could no longer be found. One explanation for this might be that the trains of impulses in paralysis are caused by a toxic substance in the haemolymph which is washed away when the haemocoel is flushed with Ringer solution.

The appearance of cockroaches which are approaching death from almost any cause resembles that in late prostration. Only when they are killed quickly, as for instance by freezing to — 10°C, do they escape desiccation. It seems likely

that as the volume of haemolymph diminishes the transport of metabolites must be seriously affected. If this is so the neuromuscular disturbances following paralysis might be due to partial depolarization of charged membranes caused by accumulation of potassium ions on their outer surfaces, the animal having no transport mechanism to remove them. There is no evidence that the trains of impulses at the stage of prostration or paralysis have exactly the same characteristics as those at tremors.

DISCUSSION

There is now a great deal of evidence from many workers that the reaction of the cockroach to bodily stress resembles the effect of DDT and some other insecticides. The stimulatory effect of DDT upon respiration of many species of insects has been reported by a number of authors. We have observed that a similar surge of respiratory activity precedes paralysis or prostration and that there is a correlation between tolerance of DDT poisoning and of physical stress. Tobias, Kollros, and Savit (1946) reported an increase in the level of acetylcholine at prostration in whole *Musca domestica* and in the nerve cord of *P. americana*. Lewis and Waller (personal communication) have confirmed the increase in the nerve cord of prostrate *P. americana* and have further shown that a similar increase occurs at paralysis. Toxic substances other than the applied insecticides have been demonstrated in the haemolymph of cockroaches subjected to DDT poisoning and to mild electric shocks (Sternberg and Kearns, 1952; Sternberg, Chang, and Kearns, 1957), to pyrethrum poisoning (Blum and Kearns, 1956), to physical stress and to electric shock (Beament, 1958).

It is suggested that whenever a lethal dose of a convulsant insecticide is applied to a cockroach, unless the toxic action is very rapid, a generalized stress syndrome will be superimposed on the specific response to the insecticide and may even obscure it. It is further suggested that any stimulus, either poison or stress, which is sufficiently strong, sets up a trigger reaction at point A (see Fig. 1). The stress syndrome then commences. There is a surge of respiration which continues until the time corresponding to point B in Fig. 1. At this point either a failure of transport due to desiccation or a biochemical lesion takes place, and the insect is no longer able to make use of its food reserves. The level of high energy phosphates falls and collapse ensues.

Winteringham (1956) has shown that in prostrate houseflies (corresponding to point B in Fig. 1b) there is a fall in the level of adenosine triphosphate (ATP) in the thorax. This occurs before the exhaustion of endogenous reserves, as indicated by measurements of oxygen consumption. A similar fall in ATP at prostration has been suggested by experiments on the cockroach nerve cord (Heslop and Ray, 1958) and the observations on total oxygen consumption to the point of paralysis or prostration made in this study also support the contention that collapse of the insect is not due to exhaustion of reserves.

Acknowledgements—The authors are indebted to Dr. J. W. L. Beament, Mr. S. E. Lewis and Mr. F. P. W. Winteringham for much valuable discussion.

REFERENCES

BEAMENT J. W. L. (1958) A paralysing agent in the blood of cockroaches. *J. Ins. Physiol.* **2**, 199–224.

BLUM M. S. and KEARNS C. W. (1956) Temperature and the action of pyrethrum in the American cockroach. *J. econ. Ent.* **49**, 862–865.

HESLOP J. P. and RAY J. W. (1958) Phosphorus compounds of cockroach nerve and the effect of DDT. *Biochem. J.* **70**, 16P.

HOSKINS W. M. and GORDON H. T. (1956) Arthropod resistance to chemicals. *Ann. Rev. Ent.* **1**, 89–122.

HOYLE G. (1953) Potassium ions and insect nerve muscle. *J. exp. Biol.* **30**, 121–135.

STERNBERG J., CHANG C. S., and KEARNS C. W. (1957) DDT-induced toxins in insect blood. *Fed. Proc.* **16**, 124–125.

STERNBERG J. and KEARNS C. W. (1952) The presence of toxins other than DDT in the blood of DDT-poisoned roaches. *Science* **116**, 144–147.

TOBIAS J. M., KOLLROS J. J., and SAVIT J. (1946) Acetylcholine and related substances in the cockroach, fly and crayfish, and the effect of DDT. *J. cell. comp. Physiol.* **28**, 159–182.

WINTERINGHAM F. P. W. (1955) *Pest Infestation Research* 1955, p. 48. H.M. Stationery Office, London.

WINTERINGHAM F. P. W. (1956) Resistance of insects to insecticides. *Chem. Ind., Lond.* **1956**, 1182–1186.

VOL.
3
1959

J. Ins. Physiol., 1959, *Vol.* 3, *pp.* 402 *to* 413. *Pergamon Press Ltd., London. Printed in Great Britain*

CHEMICAL CONSTITUENTS OF HAEMOLYMPH AND TISSUE IN *TELEA POLYPHEMUS* CRAM. WITH PARTICULAR REFERENCE TO THE QUESTION OF ION BINDING*

C. B. CARRINGTON and S. M. TENNEY

Department of Physiology, Dartmouth Medical School, Hanover, New Hampshire, U.S.A.

(*Received* 13 *April* 1959)

Abstract—Normal neuromuscular function in those insects with high extracellular fluid potassium concentrations has prompted theories which state that the tissue environment is either protected by a sheath which serves as a diffusion barrier, or that the physiologically active (ionized) potassium is present in much lower concentration than the total measured, because a large fraction is 'bound'. The present study was designed to test both chemically and physiologically the possibility that ion binding occurs in haemolymph.

Analyses of the major haemolymph constituents in *Telea polyphemus* Cram. revealed a pattern similar to other Lepidoptera, the principal features being a high potassium, high magnesium, low sodium, and a low chloride concentration. Heart and wing muscle analyses indicated about twice as much potassium intracellularly as extracellularly, but there was no sodium gradient. Haemolymph ultrafiltration demonstrated that between 15 and 20 per cent of calcium and magnesium were bound to macromolecules that did not pass the ultrafilter, but no binding of either potassium or chloride was apparent. Electrophoretic analysis indicated that the principal protein migrated like an α-2-globulin, a protein with known low binding capacity. Haemolymph osmotic pressure measured with a physical method was about twice the value determined with the red cell osmometer technique, due to the rapid passage of osmotically active particles, as well as water, across the red cell membrane. These particles are probably amino acids, but they do not carry any measurable potassium with them into the red cell, indicating that even if they do serve to some extent as anions in haemolymph the complex with potassium is not sufficiently tight to be called 'bound'.

Trans-membrane resting potentials of skeletal and heart muscle determined with the micro-electrode technique were in the range predicted from the cellular and haemolymph concentrations of potassium. Heart muscle action potentials ranged from 30 to 50 per cent of the resting value and in no instance exhibited 'overshoot'. The depression in excitability of frog skeletal muscle exposed to insect haemolymph was similar to that measured in control muscles exposed to Ringer's solution with equivalent potassium concentrations. Since each of these physiological measurements is dependent solely on the concentration of ionized potassium, it may be inferred that no haemolymph potassium is in 'bound' or un-ionized state.

IT has long been known that the haemolymph of phytophagous insects is characterized by an ionic pattern which by vertebrate standards is unusual. Of particular interest has been the very high extracellular fluid potassium concentration with low sodium and chloride, high magnesium, and slightly elevated calcium

* Supported in part by a research grant from the National Heart Institute of the National Institutes of Health, Department of Health, Education, and Welfare.

concentrations. Other unusual aspects include an extraordinary high amino acid content and a total osmotic pressure which has been reported to range from values approximately equal to that of mammalian plasma (BACKMAN, 1912) to twice that value (BUCK, 1953). There is no literature on the chemical anatomy of tissues in those insects with the highest potassium values in haemolymph; nor is it known whether there is a significant change between pupal and adult haemolymph, although there is information comparing larval and pupal values (TOBIAS, 1948).

The physiological consequences of an extracellular fluid of this ionic composition, particularly upon excitable tissues, has been the subject of several investigations and considerable speculation (BARSA, 1954; HOYLE, 1957; WOOD, 1957). The remarkably high potassium concentration has been of primary interest since in most vertebrate tissues the concentration normal to the phytophagous insects would exert a profound depolarizing effect with resultant reduction of excitability. Various hypotheses have been advanced to account for the normally functioning excitable tissue in those invertebrates with a high extracellular potassium. These have included: (a) a protective sheath which acts as a diffusion barrier to the ionic species in the extracellular environment, and/or (b) cation 'binding' to an extent which reduces the concentration of potassium in ionized, or physiologically active, form to approximate the normal vertebrate range.

VOL.
3
1959

The present study was designed to examine the chemical constituents of both the haemolymph and muscle tissue in the moth *Telea polyphemus* Cram. with attention devoted particularly towards ascertaining the extent of ion binding. Specifically this study contains chemical analyses of whole serum and ultrafiltrates. Two physiological techniques were employed to evaluate the effective potassium concentration in the haemolymph: (1) measurement of the resting trans-membrane potential of muscle fibres bathed in the endogenous haemolymph, and comparison of the measured value with the theoretical value predicted by the Nernst equation assuming the analysed potassium to be totally ionized; (2) a study of the effect of insect haemolymph in altering the excitability of frog muscle. In addition, a comparison has been made between the osmotic activity of insect haemolymph as determined by a physical method and the older method utilizing the human red cell as an osmometer in order to resolve the widely varying values cited in the literature.

METHODS

Pupal haemolymph was collected in late May and early June. The haemolymph of two pupae was combined for each aliquot, centrifuged, and the supernatant fluid removed for analysis. Adult haemolymph was obtained from animals 2–4 hr after emergence. The adult was placed in a humidified chamber, the wings severed, and haemolymph collected directly into micro-pipettes from the bleeding vessels. Each analysis was from a single animal. Wing muscle and heart muscle were removed from five adult specimens, blotted, weighed, and baked. They were then reweighed, fat extracted, and ashed. Analyses for sodium and potassium were made on the ash in solution.

Sodium and potassium were measured with an open flame photometer with internal standard. Magnesium was determined with a spectrophotometer by the CLARK and CRAIG (1953) modification of the Taras Brilliant Yellow colorimetric method. Calcium was determined according to BACHRA, DAUER, and SOBEL (1958) with the following modifications: (a) samples were ashed before titration since the strong colour of the serum masked the end point when samples were titrated directly; (b) titrations were performed in a Pyrex spot plate rather than in test tubes; (c) EDTA was standardized against calcium standards of 12 mEq Ca/l. with and without 60 mEq Mg/l. The addition of magnesium to the standard caused an error (compared to the pure standard) of less than 1 per cent. The standard value determined by the mixed standard was used in all calculations. As reported by PATTON and REEDER (1956) the end point was sharper when magnesium was present. Chloride was determined by the CARAWAY and FANGER (1955) modification of the Schales and Schales titration. Inorganic phosphate was determined according to Fiske and Subbarow, and protein and NPN by the micro-Kjeldahl method (all three as cited in HAWK, OSER, and SUMMERSON 1954). pH was measured at room temperature with a glass electrode. Urea was determined by the VAN SLYKE (1927) gasometric method. Water content was determined by drying to constant weight at 105–110°C.

Osmotic pressure of pupal haemolymph was measured by the thermo-electric vapour pressure method (BALDES, 1934) and by the mammalian red cell method (BACKMAN, 1912), the latter modified as follows. One volume of packed human red cells was equilibrated with four volumes of haemolymph. The tubes were centrifuged and most of the supernatant was removed. After resuspending the cells in the remaining supernatant the haematocrit ratio (micro method) and Drabkin's haemoglobin were determined, and mean corpuscular haemoglobin concentration (MCHC) was calculated. Cells from the same subject were also equilibrated with sodium chloride solutions ranging from 0·85 to 1·8 g per cent, and with potassium chloride solutions osmotically equivalent. This method enabled estimation of the 'effective' osmotic pressure with an accuracy of $\pm$ 2 per cent. Potassium was determined in the supernatants and in the remaining cell suspensions. Potassium in milliequivalents per litre of cells was calculated from the haematocrit. No correction was applied for trapped plasma (saline), which accounts for the slight difference in calculated potassium between cells in sodium chloride and cells in high potassium solutions.

Specific gravity was determined at 24°C by weighing 100–150 λ volumes of haemolymph and ultrafiltrate in calibrated micro-pipettes. Ultrafiltrates were prepared by applying 20 cm Hg positive pressure against collodion and cellophane dialysis membranes. No differences could be detected between samples prepared with the two types of membrane. Filtration time varied from 2–4 hr. During the entire filtration time both the whole serum and ultrafiltrate sides of the membrane were in contact through air with sucrose solution isosmotic with the haemolymph.

Trans-membrane resting potentials of heart and flight muscle were measured with the intracellular micro-electrode technique of LING and GERARD (1949) in

both adult and pupal specimens. The specimen was incised, sufficiently only to expose the muscle, and pinned on a paraffin block. In study of cardiac muscle it was necessary to dissect away some of the adipose tissue to obtain adequate exposure. Measurements were made immediately after preparation and without addition of any saline or other foreign material.

Square wave stimuli of variable amplitude and duration were used to determine threshold of local excitation of paired excised frog sartorius muscle. One of each pair of muscles was equilibrated for 15 or 70 min with Ringer's solution containing 3 mEq K/l. The second muscle of each pair was equilibrated for 70 min with (a) Ringer's solution containing 10 mEq K/l., or (b) Ringer's solution containing 20 mEq K/l., or (c) for 15 min with a 1:1 distilled water dilution of pupal haemolymph. The Ringer's solution was as follows: NaCl plus KCl, 83 mM/l.; $MgSO_4$, 1 mM/l.; $CaCl_2$, 2 mM/l.; NaH_2PO_4, 5 mM/l.; Na_2HPO_4, 10 mM/l.; $NaHCO_3$, 3 mM/l. Six per cent carbon dioxide in oxygen was bubbled through all four solutions prior to and during muscle equilibration. The pH of all four solutions was 6·5 (measured).

VOL. 3 1959

RESULTS

Tables 1 and 2 list the results of chemical analyses of cell-free pupal and adult haemolymph. The pupal values do not differ greatly from those reported by others. Although the extent of examination of adult fluid was limited due to the small amount obtainable, it does not appear that there is, for those constituents measured, a significant difference following maturation. There is, however, an extraordinarily wide range of individual variation for most of the electrolytes. Table 2 also includes values for the sodium and potassium concentrations in the skeletal and heart muscle fibres from pooled tissue samples, and it appears that for potassium at least, the intra- to extra-cellular potassium gradient, although of a low order of magnitude, is in the same direction as in vertebrates. The sodium concentration

TABLE 1

	Pupal haemolymph*
Na	2·5 mEq/l.
K	59·3 mEq/l. (45·3–78·0)
Mg	73·8 mEq/l. (58·8–82·5)
Ca	11·6 mEq/l. (8·2–15·6)
Cl	19·5 mEq/l. (16·9–21·8)
P	35·1 mEq/l. (12·2–66·9)
H_2O	92 per cent
Protein	8·1 g per cent
NPN	36·5 mg per cent
Urea	7 mg per cent
pH	6·5
f.p.	−1·17°C

* Values given are means of analyses on five specimens. Maximal and minimal values are indicated in parentheses.

is so low as to be of questionable accuracy, particularly in the tissue analyses, in which case no attempt was made to correct for extracellular fluid contamination.

TABLE 2

Haemolymph*	Adult
K	54·1 mEq/l. (44·3–81)
Mg	72 mEq/l. (65·5–78·4)
Cl	20·8 mEq/l. (17·2–23·3)
Heart muscle†	
Na	20·0 mEq/kg wet weight
K	88·7 mEq/kg wet weight
Striated muscle†	
Na	17·6 mEq/kg wet weight
K	77·3 mEq/kg wet weight

* Analyses on five specimens. Range in parentheses. † Analysis of pooled tissue from five insects.

Due to the very low sodium concentration in haemolymph, it was not possible to obtain an adequately large sample of adult haemolymph for sodium analyses.

In Fig. 1 are illustrated the concentrations of the four primary electrolytes analysed in haemolymph sera and in the ultrafiltrates, together with the percentage of serum concentration appearing in the ultrafiltrate. Although, as above noted, the individual variation in concentration of any single electrolyte is great, the percentage of serum concentration appearing in the ultrafiltrate encompasses a much narrower range.

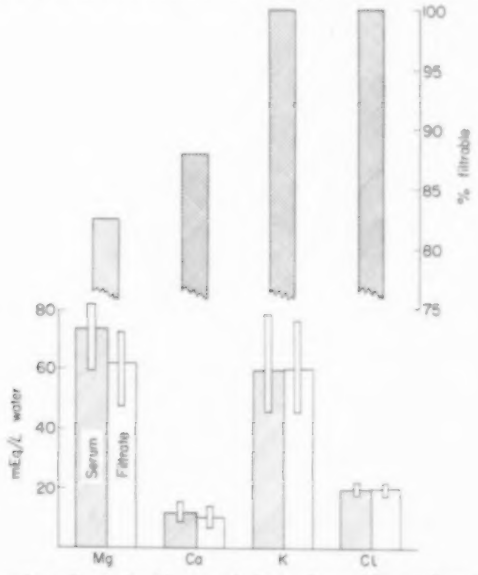


FIG. 1. Concentration of principal inorganic ions of pupal haemolymph in cell-free serum and ultrafiltrate. Range of maximum and minimum values indicated by insert bars.

Doubling the calcium concentration of haemolymph by adding calcium chloride changed the fraction bound of neither magnesium nor calcium. This indicates that the absolute amount of calcium 'bound' to non-filtrable molecules was increased two-fold, and since the concentration of protein and other macromolecules was not changed, binding sites do not appear to be saturated at physiological calcium concentrations in natural haemolymph. The effect of pH on the cation binding by haemolymph protein has not been examined in detail, but one would expect an increase as the pH rises. Since the haemolymph measurements in this study were made at pH 6·6–6·8, slightly above physiological range, the extent of binding under normal circumstances *in vivo* may be even less. It is to be noted that there was no evidence of either potassium or chloride binding. It thus appears that despite the very high protein content of haemolymph, binding to macromolecules is of some importance for divalent cations but is not demonstrable for monovalent cations.

The osmotic pressure of pupal haemolymph, as determined by freezing point depression, is equivalent to 1·76 per cent saline (14 atm); yet when measured by the human red cell osmometer technique it appears to be equivalent to 1·03 per cent sodium chloride (8·2 atm), a value 40 per cent lower than the true value (Fig. 2). This suggests that the human red blood cell membrane is permeable to

VOL. 3 1959

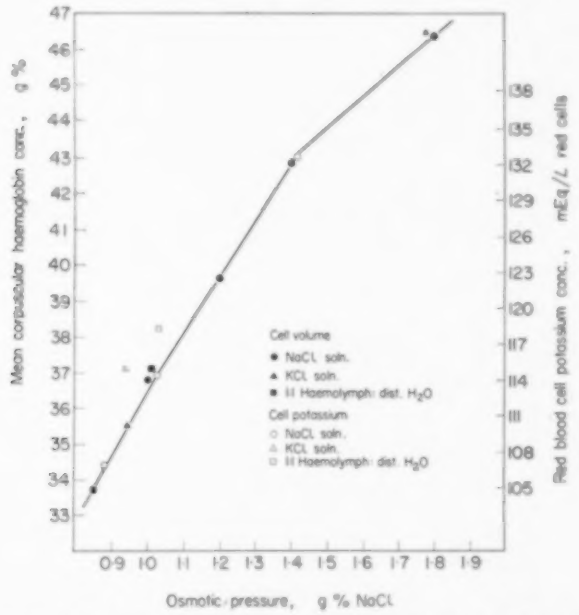


FIG. 2. Human red cell volume change following equilibration with solution of known osmotic strength and with pupal haemolymph diluted 1 : 1 with distilled water (abscissa and left ordinate). Potassium concentrations in red cells is indicated on right ordinate. The potassium values for cells suspended in high potassium solutions lie off the experimental volume curve, probably due to contamination of cell sample with trapped plasma.

a large portion of the osmotically active particles of the haemolymph, and this could explain the different values for haemolymph osmotic pressure reported in the literature, since many of the earlier determinations employed the red cell osmometer technique. It is conceivable that these osmotically active particles, presumably amino acids, might serve to bind potassium, but if the ultrafilter were permeable to these particles, that technique would fail to demonstrate their binding capacity. However, if these molecules moved across the red cell membrane to establish osmotic equilibrium, and took with them any 'bound' potassium there should result a rise in intra-erythrocyte potassium concentration. Consequently, the potassium concentration and haematocrit ratio were determined using human red cells suspended in haemolymph and for comparison, in cells suspended in sodium chloride, and potassium chloride solutions of varying tonicities. Since there does result some red cell shrinkage on exposure to haemolymph or any other hypertonic solution, the normal intracellular potassium concentration would rise due to loss of cell water. In order to examine the possibility of potassium entry it is necessary to correct for this simple concentration effect. The haemolymph used in this experiment had an 'apparent' osmotic activity of 1·03 per cent NaCl and a potassium concentration of 70 mEq/l. The potassium concentration of red blood cells suspended in this haemolymph would be equal to the value predicted from loss of cell water provided that the cells neither gain nor lose potassium when suspended in the haemolymph. The predicted potassium concentration was 117·4 mEq/l., and the actually determined value was 117·6 mEq K/l. That is to say, the change in cellular potassium content is explainable as a simple concentration effect accompanying cell volume change and provides evidence that whatever particles enter the red cell from haemolymph are not accompanied by potassium (see Fig. 2).

The potential difference existing across the membrane of a living cell is explainable in terms of the gradient of chemical potential, and since the latter is a function of ions in solution it would be instructive to compare the measured value with that theoretically predictable from the measured chemical constituents. Since the membrane potential is almost entirely accounted for by the potassium gradient, if the measured value is equal to the predicted, then this may be taken as reasonable evidence that the potassium exists in ionized form. It is interesting that resting membrane potentials in insect tissue which have been reported previously have all been measured in artificial solutions and hence do not provide pertinent information relative to the question of potassium state in haemolymph.

Trans-membrane resting potentials measured on the striated and cardiac muscle in late pupae gave a mean value of 15·4 and 18·3 mV respectively. In a one-week-old adult the cardiac trans-membrane potential was 8·5 mV. The range of potassium values listed in Tables 1 and 2 provide data for calculating predicted membrane potentials by the Nernst equation and indicate that the values should lie in the range of 8–18 mV. The calculated potentials entail two chief sources of error: (a) the contribution of ions other than potassium have been neglected, a practice commonly accepted with vertebrate tissues, but possibly

erroneous with insect; and (b) tissue analyses were not corrected for extracellular fluid contamination. The true intracellular potassium concentration is probably somewhat higher than reported. Nonetheless, the measured values are in remarkably close agreement with those predicted and provide further evidence that the potassium in the haemolymph is in ionized form.

Trans-membrane action potentials from pupal heart muscle were never equal to the resting potential but were in the range 30–50 per cent of the resting value. In all instances there was incomplete depolarization and no evidence of 'overshoot', phenomena very similar to that seen in vertebrate smooth muscle.

Excitability of frog skeletal muscle as measured by the strength–duration curve shows a progressive decrease as the extracellular fluid potassium concentration is elevated. A muscle bathed in haemolymph diluted 1:1 with distilled water, the resulting solution containing 30·4 mEq K/l., reacted as expected for an ionic potassium concentration greater than 20 mEq/l. (Fig. 3). This would suggest that little or none of the potassium present in haemolymph was in a non-active state. However, the low sodium and chloride content of the diluted haemolymph prevent exact interpretation.

VOL. 3 1959

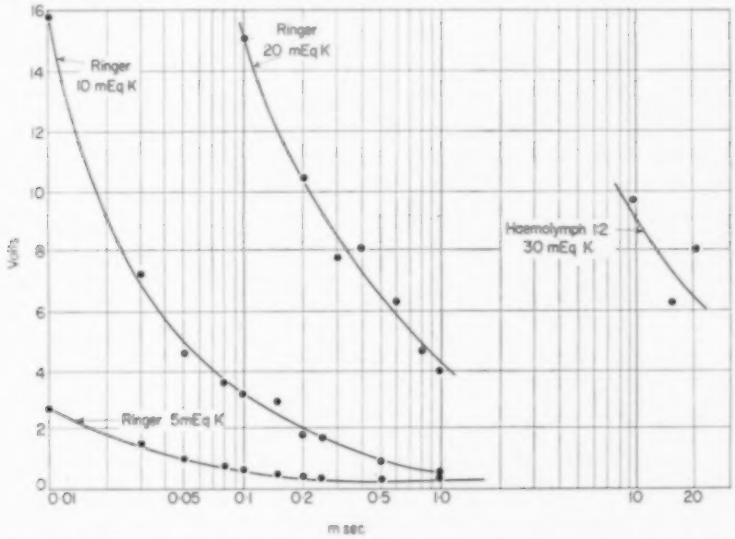


FIG. 3. Excitability curve of frog sartorius muscle bathed in Ringer's solutions of varying potassium concentration and in haemolymph diluted 1 : 1 with distilled water.

DISCUSSION

Since the earliest analyses of insect haemolymph, and especially since the development of the ionic theory of membrane potentials, there has been a great deal of speculation concerning ion binding in insect haemolymph. The pertinent literature prior to 1953 has been reviewed by BUCK (1953). There has never been any direct evidence for ion binding in haemolymph, the conclusion rather having

appeared necessary from certain experiments that appeared difficult to interpret otherwise. BARSA (1954), for instance, in studying isolated heart preparations of *Chortophaga viridifasciata* and *Samia walkeri* bathed in a variety of solutions, concluded that half the potassium and most of the magnesium occurring in natural haemolymph must be bound because optimal heart function was achieved in artificial solutions made up of chloride salts of the various cations only when the cation ratios were much lower than in haemolymph. In addition to the difficulty of accepting such indirect and inferential evidence, the fact that attempts to demonstrate potassium binding in other biological systems (STEINBACH, 1950) have not been successful should provoke serious scepticism. The experimental procedure may have included details which themselves were responsible for the observations noted without introducing the issue of ion binding. These include the use of chloride as the anion species in making artificial bathing solutions whereas sulphate, phosphate, organic acids, and other organic compounds may serve this function in natural haemolymph. Although the effects of anions on membrane phenomena have not been studied in detail, it is possible that the chloride ion has some specific effect (FALK and MCGRATH, 1958). Secondly, ion antagonism is certainly an important phenomenon in insect physiology, but this cannot be evaluated simply by varying the ratio of cations without concern for their absolute value. WOOD (1957) found that the sodium concentration had an effect on both resting and action potentials of *Carausius morosus* and that magnesium above a certain absolute value decreased the action potential and could not then be corrected by calcium antagonism. And finally, the use of sucrose as an inert constituent to elevate the osmotic pressure in insect solutions may introduce specific alterations since it is known that sucrose is toxic to vertebrate muscle (CARLSON, 1906).

On the other hand, there has also been considerable indirect evidence suggesting that potassium is for the most part in physiologically active form. HOYLE (1953, 1957) and TWAROG and ROEDER (1956) have found evidence for an active ion-barrier sheath enclosing the nerves. Thus, 70 mEq of potassium per litre were tolerated by nerves of the locust as long as the natural sheath remained intact. Removal of the sheath led to an immediate depolarization. HOYLE has also reported that a rapid rise in the haemolymph potassium concentration to a peak of 80 mEq/l. occurs in the locust at moulting and during this time muscular contraction is very feeble. Electrical recording suggested a partial neuro-muscular block to be present. He considered both the weakness and the sub-neuro-muscular block to be of the degree expected with a free ionic potassium value of about the concentration measured in the serum. Further evidence tending to support the hypothesis that in the insect potassium is in the ionized form is provided by the experiment of RAMSAY (1952) in which insects fed blood containing potassium chloride became weaker, and finally were unable to function as potassium was absorbed (potassium eventually went as high as 124 mEq/l.).

Speculation on cation binding has been closely associated with attempts to discover the anion components of the haemolymph not accounted for by chloride,

phosphate, and sulphate. The three most likely possibilities have been proteins, amino acids, and organic acids. The haemolymph protein concentration of many insects is relatively low in contrast to mammalian serum. FLORKIN (1936) states that the average plasma protein content is about 5 per cent in Hymenoptera, 3–4 per cent in Coleoptera, 2 per cent in Lepidoptera, and 1 per cent in Orthoptera. Our measurements on the Lepidopteran, *Telea polyphemus*, indicate a higher value, about 8 per cent. Amino acids are present in high concentration in insect haemolymph and account for approximately 50 per cent of the osmotically active particles in some cases (BUCK, 1953). Consideration of the isoelectric point of amino acids of insect haemolymph indicates that these molecules would not make a substantial contribution to the anion pool, and our data indicating free movement of these substances across the red cell membrane without associated potassium suggest very strongly that potassium does not complex with amino acids sufficiently tightly to be called 'bound'. Organic acids have been found in significant amount in several insects, and the ability of such organic acids as fumarate and citrate to form poorly dissociated complexes with divalent ions is well known, and the possibility that organic acids cause an appreciable depression of the ionized calcium and magnesium components in insects deserves further study. It seems, however, unlikely that the same may be true of potassium.

There are relatively few determinations of sulphur. LEVENBOOK (1950) found in *Gastrophilus* larvae 9·7 mg per cent of sulphur of which 6·8 mg per cent was inorganic. BUCK (1953) quotes Barber as finding 30 mg per cent in *Prodenia*. This would represent about 12 mEq sulphate ion if the ratio of total to inorganic were the same as in *Gastrophilus*. Values for phosphorus concentration cover a wide range, some being as high as 67 mg per cent (BUCK, 1953). We have found concentrations in *Telea polyphemus* all the way from 38 to 270 mg per cent, but these values may be excessive since no attempt was made to inhibit phosphatases. The total value was about twice the inorganic value. It is very likely that sulphate and phosphate compounds with divalent metallic cations are not entirely dissociated. WALSER and BROWDER (1958) state that the dissociation constant at 40°C and in ionic strength of 0·15 is 0·004 for calcium sulphate and 0·050 for magnesium sulphate. We have not attempted to analyse haemolymph for sulphate but were it at a level comparable to that of *Prodenia* it can be seen that sulphate and phosphate together could account for a considerable decrease in the ionic activities of calcium and magnesium ion in this plasma. As with the organic acids, however, it is doubtful that the same is true for potassium.

Were a significant amount of the haemolyph potassium in bound form, it would be anticipated that there would be a major discrepancy between the measured muscle membrane potential and the potential predicted by the Nernst equation using ion concentration values determined by chemical analyses. We have been unable to find in the literature reports of the resting membrane potential of muscle while bathed in the natural fluids. BELTON (1958) has reported resting membrane potentials of 40–65 mV in four moth species when the muscles were bathed in two different artificial solutions containing 10 and 50 mEq K/l. respectively.

VOL. 3 1959

He found no difference in potential between the two solutions and concluded that a diffusion barrier was present. He noted, however, that large changes in the concentrations of various cations effect the muscles after a considerable delay, but no further details were reported. A diffusion barrier would not protect the muscle *in vivo* except against transient changes in haemolymph concentrations. It is not clear from BELTON's brief report whether or not the potentials recorded were considered to correspond to those in the natural state, and certainly the solutions were not physiological. It is otherwise difficult to reconcile his findings with our own. Measurements of resting membrane potential fall within the general range predicted by the Nernst equation, thus adding further evidence that the haemolymph potassium is in an active state from the physical point of view. Physiologically it would appear that whatever ion antagonism may be present in insect haemolymph, it does not act in such a manner as to alter the expected trans-membrane potential.

Such low membrane potentials in vertebrate tissue would probably not be compatible with a regenerative response or mechanical contraction. In the insect however, it has been shown (HAGIWARA, 1953; HOYLE, 1957) that contraction occurs in high potassium solutions without a propagated action potential along the muscle surface. The unique anatomical arrangement of multiple end plates on insect skeletal muscle provides for almost instantaneous depolarization of the entire unit making conduction unnecessary. In heart muscle the extremely slow conduction velocity (TENNEY, 1953) does not demand a large potential and the propagated disturbance here has many similarities to vertebrate smooth muscle. In nerve, however, the rapid conduction demands greater membrane potentials and presumably these are achieved by the action of the active ion-barrier sheath surrounding the nerves as demonstrated by HOYLE (1953, 1957) and by TWAROG and ROEDER (1956).

REFERENCES

BACHRA B. N., DUAER A., and SOBEL A. E. (1958) The complexometric titration of micro and ultramicro quantities of calcium in blood, serum, urine, and inorganic salt solutions. *Clin. Chem.* **4**, 107–119.

BACKMAN E. (1912) Der osmotische Druck bei einigen Wasserkäfern. *Zbl. Physiol.* **25**, 779–782.

BALDES E. J. (1934) A micro method of measuring osmotic pressure. *J. sci. Instrum.* **11**, 223–225.

BARSA M. C. (1954) The behaviour of isolated hearts of the grasshopper, *Chortophaga viridifasciata*, and the moth, *Samia walkeri*, in solutions with different concentrations of sodium, potassium, calcium, and magnesium. *J. gen. Physiol.* **38**, 79–92.

BELTON P. (1958) Membrane potentials recorded from moth muscle fibres. *J. Physiol.* **142**, 20–21P.

BUCK J. B. (1953) Physical properties and chemical composition of insect blood. *Insect Physiology*, edited by ROEDER K. D. Wiley, New York. pp. 147–190.

CARAWAY W. T. and FANGER H. (1955) Ultramicro methods in clinical chemistry. *Amer. J. clin. Path.* **25**, 317–331.

CARLSON A. J. (1906) On the cause of the cessation of the rhythm of automatic tissues in isotonic solutions of non-electrolytes. *Amer. J. Physiol.* **16**, 221–229.

CLARK E. W. and CRAIG R. (1953) The calcium and magnesium content in the haemolymph of certain insects. *Physiol. Zool.* **26**, 101–107.

DRILHON A. (1934) Sur le milieu interieur des Lepidoptères. *C.R. Soc. Biol., Paris* **115**, 1194–1195.

DUVAL M., PORTIER P., and COURTOIS A. (1928) Sur la presence de grandes quantités d'acides amines dans le sang des insectes. *C. R. Acad. Sci., Paris* **186**, 652.

FALK G. and MCGRATH W. R. (1958) Role of anion permeation in muscle contraction. *Fed. Proc.* **17**, 44.

FARBER S. J., SCHUBERT M., and SCHUSTER N. (1957) The binding of cations by chondroitin sulphate. *J. clin. Invest.* **36**, 1715–1722.

FLORKIN M. (1936) Nouvelles données sur la teneur en protéines du plasma sanguin des Insectes. *C.R. soc. Biol., Paris* **123**, 1024–1026.

GESE P. K. (1950) The concentration of certain inorganic constituents in the blood of cynthia pupa *Samia walkeri* Felder and Felder. *Physiol. Zool.* **23**, 109–112.

HAGIWARA S. (1953) Neuro-muscular transmission in insects. *Jap. J. Physiol.* **3**, 284–296.

HOYLE G. (1953) Potassium ions and insect nerve muscle. *J. exp. Biol.* **30**, 121–135.

HOYLE G. (1956) Sodium and potassium changes occurring in the haemolymph of insects at the time of moulting and their physiological consequences. *Nature, Lond.* **178**, 1236–1237.

HOYLE G. (1957) Nervous control of insect muscles. In *Recent Advances in Invertebrate Physiology*, Univ. Oregon Publ. Eugene. pp. 73–98.

KLOTZ I. (1949) The nature of some ion protein complexes. *Cold Spr. Harb. Symp. quant. Biol.* **14**, 97–116.

LEVENBOOK L. (1950) The composition of horse bot fly (*Gastrophilus intestinalis*) larva blood. *Biochem. J.* **47**, 337–346.

LING G. and GERARD R. W. (1949) The normal membrane potential of frog sartorius fibre. *J. cell. comp. Physiol.* **34**, 388–396.

PATTON J. and REEDER W. (1956) New indicator for titration of calcium with (ethylenedinitrilo) tetra-acetate. *Analyt. Chem.* **28**, 1026–1028.

RAMSAY J. A. (1952) The excretion of sodium and potassium by the Malpighian tubules of *Rhodnius*. *J. exp. Biol.* **29**, 110–126.

STEINBACH H. B. (1950) Ion binding in muscle homogenates. *Amer. J. Physiol.* **163**, 236–246.

TENNEY S. M. (1953) Observations on the physiology of the Lepidopteran heart with special reference to reversal of the beat. *Physiol. Comp.* **3**, 286–306.

TOBIAS J. M. (1948) The high potassium and low sodium in the body fluid and tissues of a phytophagous insect, the silkworm *Bombyx mori* and the change before pupation. *J. cell comp. Physiol.* **31**, 143–148.

TWAROG B. M. and ROEDER K. D. (1956) Properties of the connective tissue sheath of the cockroach abdominal nerve cord. *Biol. Bull., Woods Hole* **111**, 278–286.

VAN SLYKE D. D. (1927) Determination of urea by gasometric measurement of the carbon dioxide formed by the action of urease. *J. biol. Chem.* **73**, 695–723.

WALSER M. and BROWDER A. (1958) Effect of sulphate on physical state and renal excretion of divalent cations (abstr.) *J. clin. Invest.* **37**, 240.

WOOD, D. W. (1957) The effect of ions upon neuromuscular transmission in a herbivorous insect. *J. Physiol.* **138**, 119–139.

VOL.
3
1959

PAPERS TO BE PUBLISHED IN FUTURE ISSUES

P. FAULKNER and B. BHEEMESWAR: Metabolism of oximes in the silkworm *Bombyx mori* L.—II. Metabolism of pyruvic oxime—2^{14}C

D. R. P. MURRAY: The stimulus to feeding in larvae of *Tenebrio molitor*

T. YAMASAKI and T. NARAHASHI: Synaptic transmission in the last abdominal ganglion of the cockroach

M. J. WAY: The effects of freezing temperature on the developing egg of *Leptohylemyia coarctata* Fall. (Diptera, Muscidae) with special reference to diapause development

H. NEMENZ: On the osmotic regulation of the larvae of *Ephydra cinerea*

L. B. BROWNE and D. R. EVANS: Locomotor activity of the blowfly as a function of feeding and starvation

K. D. ROEDER, L. TOZIAN, and E. A. WEIANT: Endogenous nerve activity and behaviour in the mantis and cockroach

M. BOCCACCI, G. NATALIZI, and S. BETTINI: Research on the mode of action of halogen containing thiol alkylating agents on insects. Effect of iodoacetic acid on choline acetylase

G. HOYLE: The action of carbon dioxide gas on an insect spiracular muscle

VOL.
3
1959